FORM PTO-1390 (Modified) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE
(REV 11-2000)

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	
522-1780	
U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.5)	
To Be Assigned 10/070018	

INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP00/08410	August 29,2000	August 30, 1999 & June 27, 2000

TITLE OF INVENTION
Novel Targer for Antiparasitic Agents and Inhibitors Thereof

APPLICANT(S) FOR DO/EO/US
THEVELEIN, Johan and VAN DIJCK, Patrick

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information.

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include itens (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S C. 371 (c)(3))
   a. ☒ are attached hereto (required only if not communicated by the International Bureau).
   b. ☒ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S C 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☐ Certificate of Mailing by Express Mail
23. ☐ Other items or information:

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
10/070018 To Be Assigned	PCT/EP00/08410	522-1780

24. The following fees are submitted:	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :		
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**		
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**		
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$890.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$0.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	24 - 20 =	4	x $18.00	**$72.00**	
Independent claims	5 - 3 =	2	x $84.00	**$168.00**	
Multiple Dependent Claims **(check if applicable)**.			☐	**$0.00**	
TOTAL OF ABOVE CALCULATIONS =				**$1,130.00**	
☒ Applicant claims small entity status. See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				**$565.00**	
SUBTOTAL =				**$565.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)) +				**$0.00**	
TOTAL NATIONAL FEE =				**$565.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐				**$0.00**	
TOTAL FEES ENCLOSED =				**$565.00**	
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of **$565.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **12-0913** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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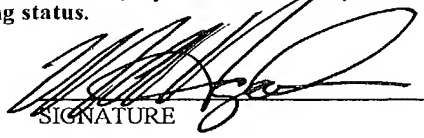

SIGNATURE

Mark A. Hagedorn
NAME

44,731
REGISTRATION NUMBER

February 27, 2002
DATE

522-1780

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE THE REISSUE APPLICATION OF)
)
Thevelein et al.)
)
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Serial No.: To Be Assigned)
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File Date: Herewith)
)
For: Novel Target for Antiparasitic Agents)
and Inhibitors Thereof)**Certificate of Mailing**

)I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to " Director of Patents and Trademarks, Washington, D.C 20231," on February 27, 2002.
Name of person signing Terri Dickinson
Signature _____

AMENDMENT ACCOMPANYING APPLICATION

Honorable Director of Patents
and Trademarks
Washington, D.C. 20231

Dear Sir:

The present Application is the National Filing of International Application number PCT/EP00/08410. Appended hereto is a copy of the International Preliminary Examination report for that Application, having appended thereto the claims as they currently appear in the International Application. Before calculation of the National Filing fee for the United States, it is requested that the Application be amended as follows:

IN THE CLAIMS:

Cancel claims 1 through 32 without prejudice, and substitute new claims 33 through 56 as

follows:

33. A method for identifying one or more candidate antiparasitic agents, comprising the steps of:

(a) contacting a candidate antiparasitic agent with a biological medium comprising (i) a phosphate selected from the group consisting of sugar-phosphates, glycerol-phosphates and sugar alcohol phosphates and (ii) a phosphate phosphatase converting the said phosphate into the corresponding unphosphorylated compound;

(b) measuring phosphate phosphatase activity in the said biological medium;

(c) repeating steps (a) and (b) with further candidate antiparasitic agents; and

(d) selecting at least one candidate antiparasitic agent which reduces phosphate phosphatase activity in the said biological medium by at least 25% compared with the said activity in the absence of the said candidate antiparasitic agent.

34. The method of claim 33, wherein the reduction of phosphate phosphatase activity by the candidate antiparasitic agent is greater than that obtained by N-ethylmaleimide or dithiodinitrobenzoate.

35. The method of claim 33, further comprising the steps of:

(e) assessing the activity of an enzyme involved in the synthesis of the said phosphate; and

(f) selecting at least one candidate antiparasitic agent which reduces the activity of the said enzyme while maintaining at least 25% of the activity of the said enzyme in the

absence of the said candidate antiparasitic agent.

36. The method of claim 33, wherein the said phosphate is selected from the group consisting of trehalose-6-phosphate, glycerol-3-phosphate, mannitol-1-phosphate, sorbitol-6-phosphate, arabitol-5-phosphate and erythritol-4-phosphate.

37. The method of claim 33, wherein the biological medium includes sub-cellular organelles or sub-cellular non-organelle components or a cell culture or a cell extract or a whole-cell preparation or an animal tissue or a plant tissue.

38. A method according to claim 33, wherein the biological medium includes sub-cellular organelles or sub-cellular non-organelle components or a cell culture or a cell extract or a whole-cell preparation which are obtained from cells of the parasite being the target of the candidate antiparasitic agent.

39. A method according to claim 33, wherein step (a) is carried out *in vitro* and wherein the method further comprises, after step (d), the steps of:

a) contacting the candidate antiparasitic agents selected in step (d) with a biological medium comprising whole-cells having the said phosphate phosphatase as an intracellular enzyme; and
b) selecting those candidate antiparasitic agents which reduce growth of the said cells.

40. A method according to claim 33, wherein the parasite being the target of the candidate

antiparasitic agent is selected from the group consisting of fungi, worms, bacteria, protozoa, nematodes, mites and insects.

41. An antiparasitic agent identifiable by a method comprising the steps of:

(a) contacting a candidate antiparasitic agent with a biological medium comprising (i) a phosphate selected from the group consisting of sugar-phosphates, glycerol-phosphates and sugar alcohol phosphates and (ii) a phosphate phosphatase converting the said phosphate into the corresponding unphosphorylated compound;

(b) measuring phosphate phosphatase activity in the said biological medium;

(c) repeating steps (a) and (b) with further candidate antiparasitic agents; and

(d) selecting at least one candidate antiparasitic agent which reduces phosphate phosphatase activity in the said biological medium by at least 25% compared with the said activity in the absence of the said candidate antiparasitic agent.

42. The antiparasitic agent of claim 41, wherein the target of the said antiparasitic agent is selected from the group consisting of fungi, worms, bacteria, protozoa, nematodes, mites and insects.

43. The antiparasitic agent of claim 41, being an antifungal agent.

44. The antiparasitic agent of claim 41, being a biocide.

45. The antiparasitic agent of claim 41, in combination with another antiparasitic agent and/or a compound that induces or enhances the stress response of cells.

46. A biologically, prophylactically or therapeutically active composition comprising a biologically or therapeutically or prophylactically effective amount of an antiparasitic agent according to claim 41, or a pharmaceutically acceptable salt or ester or pro-drug
thereof, optionally in combination with another antiparasitic agent or a stress raising factor .

47. A method of increasing , in a yeast, fungal, bacterial, protozoan, nematode, worm, mite or insect cell, the content of a phosphate selected from the group consisting of sugar-phosphates, glycerol-phosphates and sugar alcohol phosphates, the said method comprising the step of inhibiting or blocking expression of the phosphate phosphatase. converting the said phosphate into the corresponding unphosphorylated compound.

48. The method of claim 47, wherein the said phosphate is selected from the group consisting of trehalose-6-phosphate, glycerol-3-phosphate, mannitol-1-phosphate, sorbitol-6-phosphate, arabitol-5-phosphate and erythritol-4-phosphate.

49. The method of claim 47, comprising the step of contacting the said cell with an inhibitor of the said phosphate phosphatase.

50. The method of claim 47, wherein blocking phosphate phosphatase expression is effected by inactivating the gene which encodes the said phosphate phosphatase.

51. The method of claim 47, wherein blocking phosphate phosphatase expression is effected by means of a single or double knockout deletion mutation of the gene which encodes the said phosphate phosphatase.

52. A method of reducing or impairing the pathogenicity of a parasite by promoting hyperaccumulation of a phosphate selected from the group consisting of sugar-phosphates, glycerol-phosphates and sugar alcohol phosphates in the cells of the said parasite.

53. A method for preventing or treating a parasitic infection or infestation in a human or animal or plant, comprising administration to the human or animal or plant in need of such treatment of a therapeutically or prophylactically effective amount of an inhibitor of a phosphate phosphatase converting a phosphate selected from the group consisting of sugar-phosphates, glycerol-phosphates and sugar alcohol phosphates into the corresponding unphosphorylated compound, or a pharmaceutically acceptable salt or ester or pro-drug of the said inhibitor.

54. The method of claim 53, wherein the said inhibitor does not interfere with any essential metabolic process or pathway of the human or animal or plant in need of said treatment.

55. The method of claim 53, wherein the said phosphate is selected from the group consisting of trehalose-6-phosphate, glycerol-3-phosphate, mannitol-1-phosphate, sorbitol-6-phosphate, arabitol-5-phosphate and erythritol-4-phosphate.

56. The method of claim 53, wherein administration is effected topically or systemically.

REMARKS

The above Amendments are being made in order to introduce a new claim set in the U.S. Application, as well as eliminate multiple dependancy should any multiple dependency remain, that is unintended, and the Patent and Trademark Office is requested to cancel any multiple dependant claims without prejudice before calculation of the Application filing fee.

Examination of the Application on its merits is awaited.

February 27, 2002
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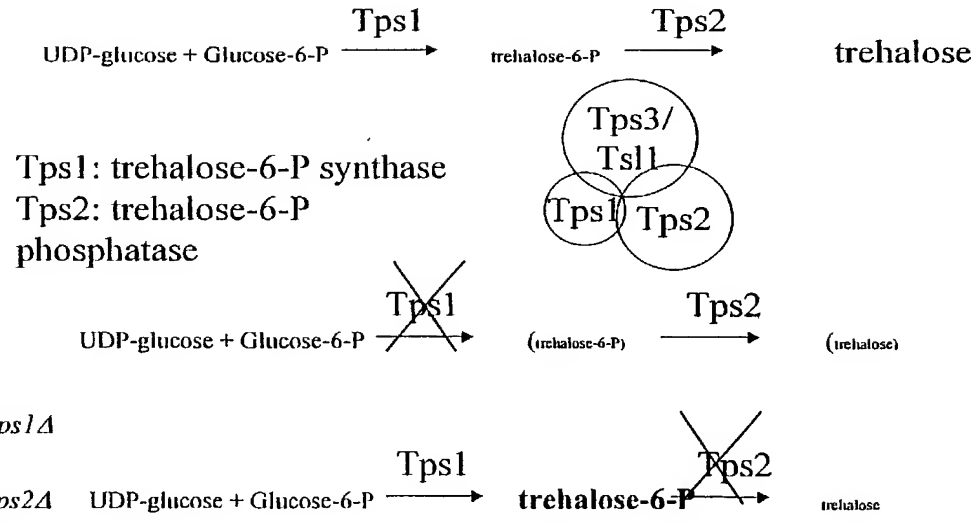
[Continued on next page]

(54) **Title:** NOVEL TARGET FOR ANTIPARASITIC AGENTS AND INHIBITORS THEREOF



(57) **Abstract:** The use of an enzyme found in fungi, bacteria, insects, nematodes, worms, mites, protozoa etc. as a target in a screening assay is described by means of which agents capable of inhibiting the function of that enzyme may be identified. The screening assay may include complete cell or purified-enzyme assays. In particular, the present invention relates to a screening assay for inhibitors or suppressors of sugar alcohol phosphatases or sugar phosphatases, and more in particular inhibitors or suppressors of trehalose-6-phosphate phosphatase, as well as preparations, in particular, pharmaceutical preparations, which include inhibitors or suppressors obtained from the screening assay. Inhibitors are described as well as applications in biocides and antifungal pharmaceuticals.

WO 01/16357 A2

NOVEL TARGET FOR ANTIPARASITIC AGENTS AND INHIBITORS THEREOF

The present invention relates to the use of an enzyme found in fungi, bacteria, insects, nematodes, worms, protozoa, mites and other organisms expressing that enzyme as a target in a screening assay by means of which agents capable of inhibiting the function of that enzyme may be identified. The screening assay may include complete cell or purified-enzyme assays adaptable for automation. In particular, the present invention relates to a screening assay for inhibitors or suppressors of sugar phosphatases or sugar alcohol phosphatases as well as preparations, in particular, pharmaceutical preparations, which include the inhibitor or suppressor obtained from the screening assay.

TECHNICAL BACKGROUND

Fungal cells and fungal spores have an amazing capacity for adaptation to survival under stress conditions and resume their vital functions as soon as the stress condition is removed. These organisms withstand freezing, strong vacuum, high doses of ionizing radiation, high pressure, osmotic stress and extreme temperatures without suffering damage and many of them accumulate the non-reducing disaccharide trehalose as a protein and membrane protectant.

The biosynthesis of trehalose consists of two enzymatic steps catalyzed by trehalose-6-phosphate synthase (TPS, expressed by the *TPS1* gene), which synthesizes trehalose-6-phosphate and by trehalose-6-phosphate phosphatase (TPP, expressed by the *TPS2* gene) which forms trehalose. Detailed information on the composition and function of the trehalose synthase complex can be found in Reinders A. et al. (Mol. Microbiol. 24, 687-695, 1997) and Bell W. et al. (J. Biol. Chem. 276, 33311-33319, 1998). In addition to its classical role in storage sugar accumulation, trehalose metabolism is known to play an important role in stress resistance, control of glucose influx into glycolysis and glucose-induced signaling.

As described in the article by De Virgilio et al., J.Biochem., vol. 212, 1993, pages 315-323, the disruption of the *TPS2* gene in *Saccharomyces cerevisiae* causes loss of trehalose-6-phosphatase activity and accumulation of trehalose-6-phosphate whereas the wild-type strain had hardly detectable levels of the trehalose-6-phosphate. It appears that accumulation of high levels of trehalose-6-phosphate is inhibitory to growth and survival,

since conditions that lead to the accumulation of trehalose in wild-type cells cause loss of viability of the *tps2* disruption mutant. This is observed for instance upon incubation at 37°C and when the cells are grown into stationary phase.

The incidence of fungal infections in patients has dramatically increased in the last 20 years (Klepser M. E. et al., Ann. Pharmacother., vol. 32, 1998, pages 1353 – 1361). Especially *Candida albicans* has become an important opportunistic pathogen. Patients suffering from immunodeficiency are especially vulnerable to infections with *Candida* and other fungi. Although *Candida albicans* is a common microorganism, found in the digestive tract and cavities of 10 to 50% of normal humans, upon a decreased resistance to infection in the host or upon administration of antibiotics for a long term or upon a surgical invasion, these microorganisms proliferate abnormally, damage tissues, and can enter the blood. Treatment is often hampered by the fact that many agents which are active against fungi also are toxic to mammalian cells, leading to a low therapeutic index and undesirable side effects in the patient.

A selection of drugs is available to combat fungal infections: Amphotericin B, Flucytosine, Ketoconazole, Miconazole, Fluconazole, and Itraconazole (Polak A., Progress in Drug Research, Vol. 49, 1997, pages 219-318). All these drugs have specific drawbacks. Amphotericin B is very efficacious because of its fungicidal action but is also relatively toxic to the patient especially to the renal functions. Flucytosine has a limited antifungal spectrum and the appearance of resistant cells is very frequent. As a result it is only used in combination with other drugs. Ketoconazole has a broad spectrum and is very useful for deep mycoses but it shows significant interactions with other drugs and causes endocrinopathies and hepatopathies. It cannot be used in immunosuppressed patients. Miconazole suffers from similar problems as Ketoconazole. Fluconazole and Itraconazole are more specific, less toxic and more efficacious than Ketoconazole and Miconazole. However Fluconazole is active against fewer fungi. Itraconazole is highly lipophilic and as a result reaches only low levels in most body fluids. As a result there is a requirement for novel antifungals with broader spectrum, higher efficacy and less side-effects.

The azole class of antifungals is also widely used in agriculture to combat plant pathogenic fungi (Adams D.J., 1997, In: Molecular genetics of drug resistance, eds. Hayes J. H., Wolf C. R., Harwood Academic Publishers , The Netherlands).

A common problem with all existing drugs is the appearance of resistance in the

fungal pathogens. The longer the drugs are in use the more resistance is observed. This requires the constant development of new antifungal drugs for which the pathogens still show great sensitivity.

The same problem applies to agriculture where a steady increase in resistance to antifungals is observed in many plant pathogenic fungi (Adams D. J., 1997, In Molecular genetics of drug resistance, eds. Hayes J. H., Wolf C. R., Harwood Academic Publishers , The Netherlands).

Some antifungal drug discovery efforts have been directed at components of the fungal cell or its metabolic pathways which are unique to fungi, and hence might be used as targets of new therapeutic agents. Ideally, these should act on the fungal pathogen without undue toxicity to host cells. Because no single approach is effective against all fungal pathogens and because of the possibility of developed resistance to previously effective antifungal compounds, there remains a need for new antifungal agents with novel mechanisms of action. An essential aspect of meeting this need is the selection of an appropriate component of fungal structure or metabolism as a therapeutic target.

Despite the increased use of rational drug design, a preferred method continues to be the mass screening of compound libraries for active agents by exposing cultures of pathogens to the test compounds and assaying for inhibition of growth. In testing thousands or tens of thousands of compounds, however, a correspondingly large number of fungal cultures must be grown over time periods which are relatively long. Moreover, a compound which is found to inhibit fungal growth in culture may be acting not on the desired target but on a different, less unique fungal component, with the result that the compound may act against host cells as well and thereby produce unacceptable side effects. Consequently, there is a need for assay or screening methods which more specifically identify those agents that are active against a certain intracellular target. Additionally, there is a need for assay methods having greater throughput, i.e., which reduce the time and materials needed to test each compound of interest.

It is an object of the present invention to provide a screening assay to identify pharmaceuticals with improved effectivity against parasites or biocides against parasitic infestation.

It is an object to provide a method of identifying useful biocides, antiparasite drugs or coagents for new and conventional antiparasite drugs or biocides, having at least one of the following properties: enhancement of efficiency, lowering of concentration

required for effective treatment and reduction of side effects of conventional drugs.

It is a further object of the present invention to provide a inhibitors useful in pharmaceutical preparations or biocides against parasite infestation or disease.

It is yet a further object of the present invention to provide biocides, antiparasite drugs or coagents for new and conventional antiparasite drugs or biocides, having at least one of the following properties: enhancement of efficiency, lowering of concentration required for effective treatment and reduction of side effects of conventional drugs.

SUMMARY OF THE INVENTION

The present invention includes a novel target for antiparasitic, especially antifungal agents, such as antifungal drugs or fungicides, that is not required for growth of the parasite under standard conditions in vitro but that is specifically required for survival of the parasite under all conditions of reduced growth or absence of growth and all other conditions which in some way deviate from optimal growth conditions and/or apply stress to the parasite. This includes the growth of the pathogen in a host organism either in the presence or absence of antiparasitic or other stress inducing agents. In accordance with the present invention the word 'parasite" refers to an organism having a biosynthetic pathway in which sugar-, glycerol- and sugar alcohol phosphates are converted to the corresponding unphosphorylated compounds and which is capable of infesting or causing a disease or discomfort in a host. Examples of such parasites are fungi, bacteria, insects, nematodes, worms, mites, protozoa.

In accordance with embodiments of the present invention trehalose-6-phosphate phosphatase and similar phosphatases converting sugar-, glycerol- and sugar alcohol phosphates to the corresponding unphosphorylated compounds as well as the genes expressing these enzymes are targets for antiparasite agents, especially antifungal agents, such as antiparasitic drugs such as antifungal drugs or fungicides, either alone or in combination with other agents, e.g. drugs inhibiting growth of, or compounds inducing stress in the cells of the pathogen. Inhibition of the enzyme in parasitic pathogens, either directly or by blocking its expression from the corresponding gene, leads to accumulation of trehalose-6-phosphate rather than trehalose. As a result, they show intracellular acidification. Without being limited by theory, the sequestration of free phosphate into trehalose-6-phosphate reduces glycolitic flux since phosphate is required at the level of

glycoaldehyde-3-phosphate dehydrogenase and since trehalose-6-phosphate inhibits hexokinase. The reduced glycolitic flux and the phosphate sequestration results in reduced ATP levels which will lower the activity of the plasma membrane H^+-ATPase, further lower the intracellular pH, the activity of the multidrug – ATPase, enhancing sensitivity to antifungal drugs and many other processes depending on efficient cellular ATP production. The cells will therefore experience even more stress, will induce the stress response even more vigorously, and will enter into a vicious circle of stress and enhanced synthesis to even hyperaccumulation of trehalose-6-phosphate as a reaction against the stress, and they will rapidly die or cease to grow. Inhibition of other parallel pathways to produce trehalose is also included within the scope of the invention so that the targeted cell has high trehalose phosphate levels and low trehalose levels thus doubly weakening the cell against attack by antiparasitic agents such as antifungal drugs or fungicides or by the immune system of the host.

The present invention includes, in one aspect, inhibitors of trehalose-6-phosphate phosphatase. Inhibition of this enzyme can lead to much faster elimination of a parasitic pathogen. This effect might be obtained by such inhibitors alone or in combination with commonly known antiparasitic agents, e.g. antifungal drugs or fungicides or other compounds or factors inducing the fungal stress response. The present invention is also applicable to organisms that synthesize large quantities of trehalose, using this sequence of enzyme reactions, or at least the dephosphorylation reaction of trehalose-6-phospahte, such as bacteria, insects, nematodes, worms, mites, protozoa etc. The present invention includes all cellular parasites of mammals which depend on trehalose synthesis for survival in the host organism, and which make use of trehalose-6-phosphate phosphatase as part of the trehalose biosynthesis pathway. Further examples include Mycobacterium tuberculosum, Synechochystis sp., Streptomyces coelicolor, Salmonella typhimurium, Encephalitozoon cuniculi. The present invention also includes the addition of a stress raising factor to enhance the inhibitor in combating the pathogen. The stress factor may be temperature, osmotic pressure, an immunological reaction of the infected host or a compound having an equivalent stress-raising effect.

Non-optimal growth conditions generally prevail during growth of parasitic, e.g. fungal, pathogens in host organisms and under these conditions the target therefore is essential. Its importance for survival increases further when antiparasitic drugs are administered to combat the infection of the pathogen. Trehalose-6-phosphate

phosphatase, the second enzyme in the biosynthesis pathway of trehalose, converts trehalose-6-phosphate into trehalose. All fungi contain trehalose, which serves as a storage carbohydrate and as a stress protectant. Because of these functions trehalose is accumulated under unfavorable growth conditions and in survival forms where it can reach very high concentrations. Trehalose-6-phosphate on the other hand is normally only found in very low concentrations and its accumulation at high levels is toxic to the cells. It is synthesized by trehalose-6-phosphate synthase, which is encoded by the *TPS1* gene. Inactivation of the *TPS2* gene, which encodes trehalose-6-phosphate phosphatase, renders fungal cells hypersensitive to stress conditions, for example, commonly used antifungals specifically under non-optimal growth conditions. As an example this is demonstrated for the fungus *Saccharomyces cerevisiae.* The genes of trehalose metabolism, including the *TPS2* gene, are also present in *Candida albicans*, an important human pathogen. Equivalent genes to, or names for *TPS2 are HOG2, PFK3, D4416, YD8554.07, YDR074W.* In addition, all existing knowledge indicates that trehalose biosynthesis uses the same enzymes, trehalose-6-phosphate synthase and phosphatase, in all fungi, including fungal pathogens of humans, mammals and other animals, and plants. Under non-optimal growth conditions and a variety of stress conditions fungal cells, including those of pathogens such as *Candida albicans*, accumulate large quantities of trehalose and often also other sugars or polyols. Inhibitors of trehalose-6-phosphate phosphatase cause accumulation of trehalose-6-phosphate. Mutants deficient in the trehalose-6-phosphate phosphatase enzyme or cells treated with the novel inhibitors in accordance with the present invention accumulate large quantities of trehalose-6-phosphate under these conditions, which is highly toxic to the cells because it is a strong acid. It acts as a pleiotropic agent impairing a wide range of essential cellular components and cellular defense systems. Because the accumulation of trehalose-6-phosphate itself is a stress condition to the cell and because the trehalose-6-phosphate is synthesized as a reaction to the stress condition, the cells enter into a vicious circle after which they finally die. The present invention also includes all similar metabolic situations, such as the conversion of glycerol-3-phosphate to glycerol, mannitol-1-phosphate to mannitol, sorbitol-6-phosphate to sorbitol, arabitol-5-phosphate to arabitol, erythritol-4-phosphate to erythritol. Glycerol is generally accumulated in fungi under osmotic stress. Depending on the species sugar alcohols are accumulated together with trehalose. This is probably the case for instance in *Aspergillus fumigatus*.

It should be emphasized that a purpose of the antiparasitic, e.g. antifungal agents in accordance with the present invention is not only to block the growth of the parasite but preferably to eradicate the parasite or to assist in its eradication. Hence, although it is useful to have an antifungal that blocks the growth of the fungus, it is much better to have an antifungal that kills the fungus. Especially in immunocompromised hosts the difference between mere inhibition of growth of the fungus and actual killing of the fungus is important since these patients will have a reduced capacity to eliminate the fungus themselves when its growth is merely inhibited.

The synthesis of trehalose is induced in fungi under a variety of stress conditions. In yeast it is part of the general stress response mechanism, which is mediated by STRE-elements in the promoter of genes involved in stress protection (e.g. heat shock proteins, catalase, *TPS1* encoding trehalose-6-phosphate synthase, etc.). Hence, if the growth of the fungus is inhibited by antifungals it may also initiate the stress response. This means that inhibitors that act on the trehalose-6-phosphate phosphatase lock the fungus into a vicious circle. Because it is stressed it reacts with the stress response mechanism of which stimulation of trehalose synthesis forms part. However, because the phosphatase is inhibited, trehalose-6-phosphate will be accumulated instead of trehalose and it will become even more stressed, inducing an even stronger stress response resulting in more toxic trehalose-6-phosphate, and so on. Since the sensitivity of fungi to antifungal drugs is in part determined by the multidrug ATPase or multidrug efflux pump, severe disturbance of ATP generation will enhance the sensitivity to antifungal drugs.

The *tps2* mutant in *Saccharomyces cerevisiae* is not only temperature sensitive but also osmosensitive. It has been isolated as an osmosensitive mutant and the gene called *HOG2*. In tissues of organisms, water availability is usually restricted and the fungal pathogens are therefore generally osmostressed. Since inhibition of trehalose-6-phosphate phosphatase renders the cells osmosensitive, the fungus will also be unable to survive because of this reason.

The present invention includes all antiparasitic agents, e.g. antifungal drugs or fungicides, that inhibit enzymes converting with a low or high degree of specificity, sugar phosphates into sugars or sugar alcohol phosphates into sugar alcohols that are accumulated in large quantities for instance, but not exclusively, under conditions deviating from the optimal growth condition or as a reaction to stress conditions.

The present invention also includes all biocides acting on insects, nematodes,

bacteria, worms, mites, protozoa and other organisms accumulating large quantities of trehalose and/or similar stress-protective sugars or sugar alcohols and inhibiting enzymes converting with a low or high degree of specificity sugar phosphates into sugars or sugar alcohol phosphates into sugar alcohols that are accumulated in large quantities for instance, but not exclusively, under conditions deviating from the optimal growth condition or as a reaction to stress conditions. The present invention includes a screening assay for identifying inhibitors which inhibit a first cell enzyme converting with a low or high degree of specificity a sugar phosphate into a sugar or a sugar alcohol phosphate into a sugar alcohol that are accumulated in large quantities by cells for instance, but not exclusively, under conditions deviating from the optimal growth condition or as a reaction to stress conditions, the inhibition being either directly of the enzyme or indirectly, e.g. by suppressing the expression of the corresponding gene; the method comprising the steps of:

Step 1: contacting a candidate inhibitor with a biological medium comprising the sugar phosphate or sugar alcohol phosphate and the first enzyme;

Step 2: measuring activity which depends upon the activity of the first enzyme;

Step 3: repeating steps one and two with further candidate inhibitors; and

Step 4: selecting those candidate inhibitors which reduce activity of the enzyme compared with the same medium without the inhibitor under the same conditions.

The inhibitor may act on the enzyme or the gene expressing the enzyme or on any other factor required for successful enzymatic conversion. The first enzyme is preferably a phosphatase which synthesizes a sugar or a sugar alcohol as a reaction to stress. The reduction in activity is preferably at least 25%, more preferably at least 50%, more preferably at least 75%, more preferably at least 85% and most preferably at least 95%. In a separate assay, the activity of the second enzyme which is involved in the synthesis of the corresponding sugar phosphate or sugar alcohol phosphate is assessed and the selecting step preferentially involves selection of inhibitors which reduce the activity of the first enzyme while maintaining a viable activity of the second enzyme. Viable activities are considered to be at least 25%, more preferably at least 50% and most preferably at least 75% of the activity of the second enzyme in the same medium under the same conditions but without the inhibitor. The activity of the inhibitor is preferably better than NEM and/or DTNB, especially when the inhibitor is contacted with the

pathogenic cells rather than *in vitro*.

The biological medium may include a pure enzyme or pure enzymes, sub-cellular organelles or sub-cellular non-organelle components (*in vitro* screening), a cell culture, or animal tissue, plant tissue or a plant or an animal (*in vivo* screening). The sub-cellular organelles or sub-cellular non-organelle components or the cell culture may be obtained from the target organism, e.g. cells from insects, worms, mites or nematodes or cells of fungi, bacteria or protozoa or any other organism with a trehalose pathway. Further examples include Mycobacterium tuberculosum, Synechochystis sp., Streptomyces coelicolor, Salmonella typhimurium, Encephalitozoon cuniculi.

The first enzyme may be one or more of trehalose-6-phosphatase, glycerol-3-phosphatase, mannitol-1-phosphatase, sorbitol-6-phosphatase, arabitol-5-phosphatase, or erythritol-4-phosphatase or any similar enzyme controlling a metabolic pathway which has an intermediary compound which is normally produced as a reaction to stress conditions and/or is toxic to the cell in high concentrations.

The present invention may provide a screening assay for an inhibitor of the first enzyme in fungi. Yeast cells are extracted by vortexing in the presence of glass beads. After clearing of the extract by low-speed centrifugation, it is desalted on a gel filtration column, i. e. Sephadex G25. The final cell extract is used to measure the activity of the first enzyme. To screen the inhibitors, different concentrations of candidate compounds are added and the residual activity of the first enzyme is measured. For example, when the first enzyme is TPP, the substrate, trehalose-6-phosphate, is added to the final cell extract and either the formation of trehalose or free phosphate is then measured. Inhibitors are selected based on their ability to inhibit TPP. Ideally, inhibitors are selected which inhibit TPP but not TPS.

Filamentous fungi may be extracted after freezing in liquid nitrogen. The frozen mycelia are extracted by grinding in a mortar. After addition of the buffer, the cell extracts are cleared by low-speed centrifugation and desalted over a Sephadex column. To screen for inhibitors, different concentrations of these compounds are added and the residual activity of the first enzyme is measured. When the first enzyme is TPP, trehalose-6-phosphate is added to the cell extracts and either the phosphate or the trehalose that is generated is measured. Inhibitors are selected based on their ability to inhibit TPP. Ideally, inhibitors are selected which inhibit TPP but not TPS.

The present invention may provide a screening assay for an inhibitor of the first

enzyme in worms, e.g. nematodes. Nematodes, or worms in general, are extracted after freezing in liquid nitrogen. The frozen nematodes or worms are extracted by grinding in a mortar. After addition of the buffer, the cells are cleared by low-speed centrifugation and desalted over a Sephadex column. To screen for inhibitors, different concentrations of candidate compounds are added and the residual activity of the first enzyme is measured. When the first enzyme is TPP, trehalose-6-phosphate is added to the cell extract and either the phosphate or the trehalose that is generated is measured. Inhibitors are selected based on their ability to inhibit TPP. Ideally, inhibitors are selected which inhibit TPP but not TPS.

The present invention may provide a screening assay for an inhibitor of the first enzyme in insects or mites (Acari). Insects or mites, or parts of the insects or mites are extracted after freezing in liquid nitrogen. The frozen insects or mites are extracted by grinding in a mortar. After addition of the buffer, the cell extracts are cleared by low-speed centrifugation and desalted over a Sephadex column. To screen for inhibitors, different concentrations of candidate compounds are added to the cell extract and the residual activity of the first enzyme is measured. When the first enzyme is TPP, trehalose-6-phosphate is added to the cell extract and either the phosphate or the trehalose that is generated is measured. Inhibitors are selected based on their ability to inhibit TPP. Ideally, inhibitors are selected which inhibit TPP but not TPS.

The present invention may provide a screening assay for an inhibitor of a first enzyme in bacteria or protozoa. Bacterial or protozoal cells are extracted by vortexing in the presence of glass beads. After clearing of the extract by low-speed centrifugation, it is desalted on a gel filtration column, i. e. Sephadex G25. The final cell extract is used to measure the TPP activity. To screen the inhibitors, different concentrations of candidate compounds are added and the residual activity of the first enzyme is measured. When the first enzyme is TPP the substrate, trehalose-6-phosphate, is added to the cell extract and either the formation of trehalose or free phosphate is then measured. Inhibitors are selected based on their ability to inhibit TPP. Ideally, inhibitors are selected which inhibit TPP but not TPS.

According to the method for the identification of enzyme inhibitors of the present invention, assays may be carried out both in whole-cell preparations or in *ex vivo* cell-free systems. In each instance, the assay target is an enzyme converting with a low or high degree of specificity a sugar phosphate into a sugar or a sugar alcohol phosphate

into a sugar alcohol that are accumulated in large quantities by cells for instance, but not exclusively, under conditions deviating from the optimal growth condition or as a reaction to stress conditions, the inhibition of which enzyme significantly attenuates cell growth or is lethal. Test compounds which are found to inhibit a target enzyme in an assay of the present invention are thus identified as potential pharmaceutically or biologically active agents. It is expected that the assay methods of the present invention will be suitable for both small- and large-scale screening of test compounds, as well as in quantitative assays such as serial dilution studies wherein the target enzyme is exposed to a range of test compound concentrations.

When the method of the present invention is carried out as a whole-cell assay, the target enzyme is an intracellular enzyme and the entire, living cells are exposed to the test compound under culture conditions in which the target enzyme is produced, e.g. during non-optimal growth, stress situations. Such conditions, including essential nutrients, optimal temperatures and other parameters, depend upon the particular fungal, bacterial, insect, nematode, worm, mite or protozoal strain being targeted. The step of determining inhibition of the enzyme (step 2 above) may be carried out by observing the cell culture's growth or lack thereof; such observation may be made visually, by optical densitometric or other light absorption/scattering means, or by other suitable means, whether manual or automated.

In the above whole-cell assay, an observed lack of cell growth may be due to inhibition of the target enzyme or an entirely different effect of the test compound, and further evaluation is required to establish the mechanism of action and to determine whether the test compound is a specific inhibitor of the target enzyme. Accordingly, and in a preferred embodiment of the present invention, the method may be performed as a paired-cell assay in which each test compound is separately tested against two different sets of cells, the first having a lower enzyme activity than that of the second and thereby being more susceptible to inhibition of the enzyme.

Compounds which are found to inhibit the first, more susceptible cells but not the second are likely to have acted specifically on the target enzyme and not via a different mechanism.

One manner of achieving differential susceptibility is by using a first cell which has diminished enzyme activity relative to that of a wild-type cell, as for example a mutant strain.

Alternatively, or in conjunction with the above, differential susceptibility to target enzyme inhibitors may be obtained by using a second fungal cell which has increased enzyme activity relative to that of a wild-type cell, as for example one which has been genetically manipulated to cause overexpression of the enzyme. Such overexpression can be achieved by placing into a wild-type cell a plasmid carrying the gene for the target enzyme.

Preferred is a method in which the differentiated target enzyme activity is produced by subjecting one set of cells to a stress or non-optimal growth situation which favors enzyme activity and a second control set without the stress or non-optimal growth promoter.

The present invention also includes any inhibitor found by any of the above screening assays. In particular, the present invention includes any inhibitor found by any of the above screening assays used in a pharmaceutical preparation either alone or in combination with an antifungal drug. The present invention also includes any inhibitor found by any of the above screening assays used in a biocide acting on insects, nematodes, worms, mites, bacteria, protozoa or other organisms accumulating large quantities of a sugar alcohol or a sugar in response to stress.

The present invention also includes a screening assay for identifying inhibitors which inhibit a first cell enzyme converting with a low or high degree of specificity a sugar phosphate into a sugar or a sugar alcohol phosphate into a sugar alcohol that are accumulated in large quantities by cells for instance, but not exclusively, under conditions deviating from the optimal growth condition or as a reaction to stress conditions, the inhibition being either directly of the enzyme or indirectly, e.g. by suppressing the expression of the corresponding gene; the method comprising the steps of:

Step 1: contacting a candidate inhibitor with a biological medium comprising the sugar phosphate or sugar alcohol phosphate and the first enzyme;

Step 2: measuring activity which depends upon the activity of the first enzyme;

Step 3: repeating steps one and two with further candidate inhibitors;

Step 4: selecting those candidate inhibitors which reduce activity of the enzyme compared with the same medium without the inhibitor under the same conditions.

Step: 5 contacting the selected candidate inhibitors with a biological medium

comprising whole cells having the first enzyme as an intracellular enzyme; and

Step 6: selecting those candidate inhibitors which reduce the growth of the cells.

DEFINITIONS

The term "intracellular inhibitor", as used herein, refers to inhibitors which are able to penetrate target cells, or which are taken up by target cells, and which exhibit inhibitory activity inside the target cell. The inability to penetrate the cell (non-permeability), rapid degradation of a compound or a conversion to inactive forms once inside the cell are possible reasons for a compound to be non-active *in vivo*.

The term "target cell", as used herein, refers to yeast, fungals, bacterial, protozoal, nematodal, worm, mite or insect cells, or cells of any other organism exhibiting enzymatic TPP activity or, more in general, the sugar alcohol phopshatases or sugar phosphatases of the present invention.

The terms "specifically", "impairing specifically" and "slowing down the growth specifically of", as used herein, refer to the fact that preferentially the inhibitor impairs the action of the phosphatases only, and more specifically TPP only, in a target cell but not in cells of a host organism infected by the target cells. As such only target cells with TPP activity will be affected in their growth. Preferentially, the inhibitors of the present invention will not interfere with any metabolic pathway of the host.

The term "host" or "host organism", as used herein, refers to a human, an animal or a plant infected by the target cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 gives a representation of the trehalose biosynthesis in *Saccharomyces cerevisiae* as a two step process.

Figures 2A and B show the growth curves of prototrophic *S. cerevisiae* wild-type and *tps2Δ* strains in the presence of different concentrations of Itraconazole at 37°C, respectively. Closed symbols: wild-type (WT); open symbols: *tps2Δ* (◆): DMSO; (∝): 10^{-8}M; (ε): 10^{-7}M, (□ •): 10^{-6}M; (x, β): 10^{-5}M Itraconazole (itra).

Figure 3 shows the growth curves of prototrophic *S. cerevisiae tps2Δ* and wild-type strains in the presence of 10^{-7} M of Itraconazole at 33°C. Closed symbols: wild-type

(WT); open symbols: *tps2Δ* (♦): DMSO; (•): 10^{-7}M Itraconazole (itra).

Figure 4 shows the growth curves of prototrophic *S. cerevisiae tps2Δ* (PVD23) and wild-type (PVD32) strains in the presence of 10^{-6} M of Ketoconazole at 33°C. Closed symbols: wild-type (WT); open symbols: *tps2Δ* (♦): DMSO; (•): 10^{-6}M Ketoconazole (keto).

Figures 5A and B show the growth curves of diploid prototrophic wild-type *S. cerevisiae* (PVD190) and diploid heterozygous *S. cerevisiae tps2Δ* (PVD191) strains in the presence of different concentrations of Itraconazole at 33°C. Closed symbols: wild-type (WT); open symbols: heterozygous *tps2Δ* (♦): DMSO ; (∝):10^{-8}M; (ε):10^{-7}M; (β):$3x10^{-7}$M; (▯: 10^{-6}M; (″):$3x10^{-6}$M.

Figure 6 shows the effect of Itraconazole on the growth of wild-type *S. cerevisiae* (PVD32) and *S. cerevisiae tps2Δ* (PVD23) strains on YPD plates.

Figure 7 shows the effect of osmotic and heat stress on the growth of wild-type *S. cerevisiae* (PVD32) and *S. cerevisiae tps2Δ* (PVD23) strains on YPD plates.

Figure 8 shows the alignment for maximal amino acid similarities of trehalose phosphate phosphatase derived from *S. cerevisiae* (GENBank accession number 577801) with a homologous sequence from *C. albicans* (*C. albicans* database, http://www-sequence.stanford.edu/group/candida), with indication of the 2 putative phosphatase boxes (bold italic and underlined). Identical residues are indicated by an asterisk (*). Gaps in the amino acid sequence are represented by dots (--). A colon (:) stands for strong similarity, a dot (.) stands for weak similarity. The CLUSTAL W (1.8) multiple sequence alignment software was used.

Figure 9 shows the genomic organisation of the *C. albicans TPS2* gene, *CaTPS2*, and its flanking regions (5598 bp in total) with indication of the oligonucleotide primers used to isolate and amplify the gene (FOR2 and REV2), primers used to check for deletions in the strains (e.g. 3' diag and 5' diag) and with the relevant restriction sites.

Figure 10 shows the complete cloning strategy used to obtain the *C. albicans* pUC19/*Catps2Δ::HisGURA3HisG* disruption construct.

Figure 11 shows a Southern blot analysis for 14 heterozygous *TPS2/tps2*Δ CAI4 transformants as obtainable by using the *TPS2* disruption cassette. The presence of two bands, one of 3224 and one of 2874 bp confirm the heterozygous character. 10 µg of DNA was digested with *Eco*RI and fragments electrophoresed. Hybridization was

performed with the P^{32}-labelled 579 bp *Sna*BI- *Hind*III fragment corresponding to the 3' flanking site of *CaTPS2* gene. Left: molecular weight marker VII from Boehringer; Lanes 1-14: putative heterozygous *TPS2/tps2Δ C. albicans* mutants.

Figure 12 A shows the results of a PCR analysis with a set of three primers: Diag3, Diag5 and DiagHIS4. Left: Smart ladder molecular weight marker (Eurogentec) Lanes 1-4: putative *C. albicans* double deletion transformants. 10 µl of a standard PCR reaction mixture was loaded on a 1% agarose gel. Lane 2 corresponds with a putative double deletion mutant. **Figure 12 B** shows the results of a Southern blot analysis used for the identification and verification of putative double strain mutants. 10 µg of DNA was digested with *Eco*RI and fragments electrophoresed. Hybridization was performed with the P^{32}-labelled 579 bp *Sna*BI- *Hind*III fragment corresponding to the 3' flanking site of *CaTPS2* gene Left: molecular weight marker VII from Boehringer; Lanes 1-5, 7-10: putative *C. albicans* double deletion mutants.

Figures 13 A and B show the growth curves at 41°C (closed symbols) and 43°C (open symbols) of the three different *C. albicans* strains, *TPS2/TPS2* (wild-type, SC5314), *TPS2/tps2Δ* (heterozygous deletion mutant, CC5) and *tps2Δ/tps2Δ* (homozygous deletion mutant, CC17) respectively, grown on YPgalactose (A) and YPglucose (B) medium respectively. (", •): *TPS2/TPS2*; (∝, ←):*TPS2/tps2Δ*; (ε, Δ):*tps2Δ/tps2Δ*.

Figures 14 and 15 show the inhibition of trehalose-6-phosphate activity in *S. cerevisiae* strain PVD45 by NEM and DTNB. (14) bars from left to right respectively stand for: 35 mM; 3.5 mM; 0.35 mM; 0.035 mM; 0 mM. (15) (♦): NEM; (∝): DTNB.

Figure 16 shows the screening assay in accordance with the present invention for the determination of TPP activity and inhibition thereof in cell extracts using the Biomek robotic system and screening the DIVERSet™ (Chembridge, San Diego) compound library. **(1)**: stock plates (10^{-2}M); **(2)**:working plates (10^{-3}M); **(3)**: 2 µl test compound (or DMSO or DTNB) + 148 µl mix1 (40 µl tricine buffer 200 mM pH7; 20 µl $MgCl_2$ 0.1 M; 20 µl trehalose-6-phosphate 25 mM; 68 µl H_2O) + 10 µl extract (final protein concentration of about 10 mg/ml), incubation for 30 min at 30°C in the Biomek robotic system, 1G=2 µl pure DMSO, 1H= 2 µl DTNB (10^{-3} M in DMSO); **(4)**: 15 µl of **(3)** or 15 µl of an orthophosphate (Pi) standard series (0, 0.02, 0.1, 0.2, 1 and 5mM in H_2O) + 148 µl molybdate/Zn acetate (100mM/15mM, pH5) + 37 µl ascorbic acid (10% in H_2O

(pH5), freshly prepared), 1A-1F: Pi standard series, incubation for 20 min at 30°C in the Biomek robotic system; (5) OD_{750} measurement using a SPECTRAMAX spectrophotometer.

Figure 17 shows the structure of 7 potential TPP inhibitors identified in accordance with the present invention from the DIVERSet™ (Chembridge, San Diego) compound library. The identified compounds perform equal to or better than DTNB at 10^{-5} M under the circumstances as given. Numbers refer to the number given to the compound in the DIVERSet™ compound library. **A**: compound 136794; **B**: compound 143067; **C**: compound 113596; **D**: compound 113610; **E**: compound 133207; **F**: compound 133805; **G**: compound 100764

Figure 18 shows the *in vitro* inhibitory activity on *S. cerevisiae* trehalose phosphate phosphatase of 6 different DiverSet™ compounds, tested in 7 different concentrations. Bars, from left to right respectively stand for: 0M, $3x10^{-5}$M, $1x10^{-5}$M, $3x10^{-6}$M, $1x10^{-6}$M, $3x10^{-7}$M, $1x10^{-7}$M. Compounds are: (1) 136794; (2) 109146; (3) 143067; (4) 116321; (5): 145704; (6) DTNB.

Figures 19 A and B show the growth curves at 37°C of the wild-type *S. cerevisiae* (W303.1A) strain in the presence of different concentrations (0, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M respectively) of inhibitory compounds 136794 (**A**) and 143067 (**B**). (″): wild-type PVD517 strain; (∝): compound at 10^{-8} M; (ε): compound at 10^{-7} M; (•): compound at 10^{-6} M; (β, asterisk): compound at 10^{-5} M

Figures 20 A and B show the growth curves at 43°C of a wild-type *C. albicans* strain (SC5314) in the presence of inhibitory compounds 133207 (∝), 133805 (←), 113610 (ε), DTNB (Δ), NEM (•) or DMSO (″). Test compounds were added at two different concentrations: 10^{-5}M (**A**) and 10^{-7}M (**B**).

Figures 21 A and B show the growth curves at 39°C of a *C. albicans tps2Δ/tps2Δ* strain (CC17) in the presence of inhibitory compounds 133207 (∝), 133805 (←), 113610 (ε), DTNB (Δ), NEM (•) or DMSO (″). Test compounds were added at two different concentrations: 10^{-5}M (**A**) and 10^{-7}M (**B**).

Figure 22 shows the growth curves at 43°C of a wild-type *C. albicans* strain (SC5314) in the presence of inhibitory compounds 100764 (Δ), 136794 (•), 143067 (ε), 113610 (♦) or DMSO (″). Test compounds were added at 10^{-7}M in DMSO.

Figure 23 shows percentage survival of Balb/C mice after intravenous injection

of 10^6 (A) and 10^7 (B) cells of the wild type, *tps2 /tps2* and *TPS2/tps2* strains of *Candida*.

Figure 24 shows growth of cells of the wild type (first row), *tps2 /tps2* (second row) and *TPS2/tps2* (third row) strains after incubation for different periods of time at 44 °C. The cells were spotted in serial dilutions on YPD plates.

Figure 25 shows trehalose levels after a temperature upshift in wild type *Candida albicans* (●), heterozygous *TPS2/tps2* (■) and homozygous *tps2 /tps2* (▲) cells.

Figure 26 shows trehalose-6-phosphate levels in wild type (circles), heterozygous (squares) and homozygous (triangles) *TPS2* deletion strains. Samples were taken at different time points after the shift to 37 °C (filled symbols) or 43 °C (open symbols).

Figure 27 shows growth curves at 37 °C. Wild type (circles), heterozygous (squares) and homozygous (triangles) *TPS2* deletion strains are grown in YPD containing DMSO (filled symbols), 10^{-7}M miconazole (open symbols, left panel) or 10^{-8}M miconazole (open symbols, right panel).

Figure 28 shows growth curves at 40 °C. Wild type (circles), heterozygous (squares) and homozygous (triangles) *TPS2* deletion strains are grown in YPD containing DMSO (filled symbols), 10^{-7}M miconazole (open symbols, left panel) or 10^{-8}M miconazole (open symbols, right panel).

Figure 29 shows growth curves at 43 °C. Wild type (circles), heterozygous (squares) and homozygous (triangles) *TPS2* deletion strains are grown in YPD containing DMSO (filled symbols), 10^{-7}M miconazole (open symbols, left panel) or 10^{-8}M miconazole (open symbols, right panel).

DESCRIPTION OF THE ILLUSTRATIVE EMBODIMENTS

The present invention will be mainly described with reference to trehalose-6-phosphate and yeast cells but the present invention is not limited thereto but only by the claims. In particular the present invention may find advantageous use in the control, including human, animal or plant therapeutic control of fungi, nematodes, worms, bacteria, protozoa, mites and insects.

Fig. 1 is a schematic representation of the metabolic pathway within yeasts, other fungi, bacteria, protozoa, nematodes, worms, mites (Acari), insects and other organisms producing trehalose. Trehalose is synthesized from glucose-6-phosphate and UDP-glucose, catalyzed by trehalose-6-phosphate synthase (TPS), which is encoded by the

gene *TPS1* in yeast, to form trehalose-6-phosphate which is further processed to trehalose by trehalose-6-phosphatase (TPP) which is encoded by the gene *TPS2* in yeasts. Further, additional genes *TPS3* and *Tsl1* are believed to encode proteins which only play a regulatory role.

The promoters of the *TPS1* and *TPS2* genes are highly stress dependent and TPS is very active under bad growth conditions such as during growth on non-fermentable carbon sources, during nutrient limitation, e.g. during the stationary phase and during growth at high temperatures. Inhibition of the TPP enzyme or mutation or elimination of the *TPS2* gene while leaving the TPS enzyme intact, results in an increase in the trehalose-6-phosphate levels in the cell under conditions where wild-type cells accumulate trehalose. The present inventors have been the first to determine that specific inhibition of the TPP enzyme while maintaining the activity of the TPS enzyme makes the cell more prone to attack by antifungal agents. That is, the amount of antifungal agent required to stop growth or to kill the cell is reduced. This is particularly advantageous as the commonly used antifungal agents have serious side effects and any method of reducing the concentrations having a significant therapeutic effect is valuable. The trehalose metabolic pathway is irrelevant in humans and other mammals, so that a specific inhibitor to TPP, offers the possibility of reduced, few or no side effects. The present invention therefore sets as one of its objects the development of a specific inhibitor for TPP. Accordingly, embodiments of the present invention relate to a screening assay for detecting specific inhibitors of TPP. With "specific" is meant that the inhibitor (preferably) interferes with TPP only and not with phosphatases or any essential metabolic pathway of the host, e.g. a human, animal or plant in need of treatment.

MATERIALS AND METHODS

MEASUREMENT OF TREHALOSE –6-PHOSPHATASE ACTIVITY

Methods for the measurement of trehalose-6-phosphatase activity may comprise methods for the measurement of the trehalose that is released from trehalose-6-phosphate and/or the measurement of the phosphate that is released from the trehalose-6-phosphate.

1. Preparation of the extracts :

a) cultures are grown to the desired density and are cooled quickly on ice;

b) preferably, at least 400 mg of cells are harvested by centrifugation for 3 min at 3000 rpm;

c) cells are washed twice with ice-cold distilled water;

d) cells are resuspended in 990 µl extraction buffer consisting of 968 µl of 50 mM Imidazole (Merck 1.04716 (RT)), 2 µl of 0.5 M EDTA (Tritriplex III Merck 1.08418 (RT)), 20 µl of 0.1 M $MgCl_2$ (UCB 94046076 (RT),

e) cells are transferred to screw capped tubes and an equivalent of 0.5 ml glass beads with 0.5 mm diameter are added;

f) just before extraction 10 µl of a 100 mM PMSF solution (Sigma P7626 (RT)) in methanol is added to the cell mixture ;

g) the extraction is performed in a Fastprep apparatus (BIO101) for 20 seconds at level 4, and is repeated three times with a cooling step on ice in between. Alternatively the extraction can be performed on a vortex apparatus in glass tubes (always used for large scale preparations);

h) extracts are centrifuged at 4°C for 20 min at 14000 rpm;

i) 20 µl of the supernatant is loaded on a small Sephadex G25 column, made in a blue tip containing a siliconized glass bead. The tip is filled completely with pre-equilibrated G25 Sephadex (50 mM Tricine (Sigma T-0377 (RT)) buffer (pH7). The tips are centrifuged once before application of the extract for 1 min at 800 rpm. The extract is centrifuged for 1 min at 1000 rpm;

j) 200 µl of the eluate is added to 140 µl Assay I solution consisting of 40 µl of 200 mM Tricine buffer (pH7), 20 µl of 0.1 M $MgCl_2$, 20 µl of 25 mM trehalose-6-phosphate (Sigma) and 60 µl H_2O. For the Assay I control mixture 20 µl trehalose-6-phosphate is omitted and replaced by 20 µl H_2O.

k) the assay mixture and the control mixture are incubated for 30 min at 30°C;

l) the assay mixture and the control mixture are boiled for 5 min and cooled down to room temperature.

m) centrifugation of the micro-centrifuge tubes for 5 min at 14000 rpm

For large scale screenings to identify specific enzyme inhibitors, whereby bigger amounts of yeast extracts are required, loading on small Sephadex G25 columns (step i) is replaced by loading on a superdex200 column (Amersham Pharmacia biotech). Briefly, about 500 µl of extract (protein concentration about 15 mg/ml) in extraction buffer (see

step (d) above) is loaded on a superdex200 column equilibrated with this same extraction buffer. For each run, 500 µl of sample was loaded on the column, which was then eluted at 0.5 ml/min with a total buffer volume of 35.5 ml. 750 µl fractions were collected and the FPLC fractions containing TPP activity (fractions 11-13) pooled. These pooled fractions were then concentrated on VIVASPIN columns (VIVAscience) by a 30 min centrifugation step at 3500 rpm. The final protein concentration of the extract used for screening was about 10 mg/ml (method of Lowry, J. Biol. Chem. 193, 265-275, 1951). Alternative methods for determination of protein contents are well known in the art. Further steps were as described above (steps j to m).

2. Measurement of trehalose that is released from trehalose-6-phosphate.

One method of calculating the amount of trehalose released from trehalose-6-phosphate is by performing two steps: the hydrolysis of trehalose by trehalase and the measurement of the resulting glucose by either the glucose-oxidase/peroxidase reaction, or the use of the Trinder reagent.

a) Hydrolysis of trehalose by trehalase.

The trehalase is obtained from the fungus *Humicola grisea var. thermoidea*. The organism is grown at 40°C on a solid medium containing 4% oat and 1.8% agar. The isolation and purification of the trehalase from this fungus is performed according to Neves et al. (FEBS Lett. 283, 19-22, 1991).

After preparation and centrifugation of the extracts according to the methods described above, 30 µl of the extract is transferred to new microcentrifuge tubes, and 10 µl buffer (300 mM NaAc, 30 mM $CaCl_2$, ph 5.5) and 40 µl trehalase solution (400 U/ml) is added. The mixture is incubated at 40°C for 45 min.

A trehalose standard curve of 0,1,2,4,8 and 10 mM is also analyzed.

b) Measurement of the glucose from the hydrolysis of trehalose by trehalase by oxidation of glucose by glucose-oxidase/peroxidase.

Materials:

Solution A: containing 3.75 mg glucose-oxidase (100 U/mg), 8 mg peroxidase (100 U/mg) and 2.25 ml Tris/Cl (1M pH8) adjusted to 100 ml with water.

Solution B: containing 10 mg/ml ortho-dianisidine-diHCl.

The glucose-oxidase solution is prepared just before use by mixing 1 ml of solution B with 100 ml solution A.

The method involves the transfer of 60 µl of the product of the trehalase reaction to a glass tube, adding 1 ml of the glucose-oxidase solution, incubating for 60 min at 30 °C. The reaction is terminated by adding 0.5 ml H_2SO4 (56%) to the reaction mixture. The OD of the reaction mixture is measured at 546 nm.

A glucose standard curve with 0, 1, 2, 3 and 4 mM is analyzed at the same time.

c). Measurement of the glucose from the hydrolysis of trehalose by trehalase by oxidation of glucose using Trinder reagent.

The glucose produced from the hydrolysis of trehalose by trehalase can also be measured using the Trinder reagent (SIGMA), which is based on the same principle as the glucose oxidase reaction.

The method involves the transfer of 20 µl of the end products of the trehalase reaction in a microtiter plate well, the addition of 200 µl Trinder reagent and mixing by shaking and incubating for 15 min at 30°C. The OD of the sample is determined at 505 nm in the microtiter plate reader (Spectramax).

A glucose standard curve with 0, 1, 2, 3 and 4 mM is analyzed at the same time.

d) Calculation of the TPP activity.

Following the hydrolysis of trehalose by trehalase and the measurement of the resulting glucose by the glucose-oxidase/peroxidase reaction, the TPP activity can be calculated by dividing the OD of a sample by the time of the reaction and by the protein content (expressed as nKat/g protein).

Alternatively the trehalose that is formed by the action of the trehalose-6-phosphate enzyme can be measured by the HPLC analysis on a CarboPac PA-100 anion-exchange column as described by De Virgilio et al. (Eur. J. Biochem.212, 315-2-323, 1993).

3. Measurement of the phosphate released from the trehalose-6-phosphate.

Apart from the trehalose, the phosphate can also be measured that is released by the action of the trehalose-6-phosphate phosphatase enzyme.

This can be performed by either one of the following methods:

a) Continuous phosphate measurement using the EnzChekTM Phosphate Assay Kit (Bioprobes, Molecular Sondes). In the presence of free Pi, the substrate 2-amino-6-mercapto-7-methylpurine riboside (MESG) with maximum absorbance at 330 nm, is converted by the action of the enzyme purine nucleoside phosphorylase (PNP) to a product with maximum absorbance at 360 nm (2-amino-6-mercapto-7-methylpurine). The increase in absorbance at 360 nm is monitored spectrophotometrically and is proportional to the phosphate consumption by the MESG/PNP reaction.

b) Free phosphate measurement by the method of Bencini (Anal. Biochem. 132: 254-258, 1983). The reagent solution, an aqueous mixture of ammonium molybdate (100 mM) and zinc acetate (15 mM) at pH 5, produces a stable complex with orthophosphate (Pi). This complex is reduced by the addition of ascorbic acid (10% in H_2O (pH5), freshly prepared), whereby a product is formed that absorbs strongly at 850 nm. Measurements can be performed equally well at 750 nm. The method is linear up to 300 µM phosphate.

c) The measurement of phosphate according to the method of Fiske-Subbarow comprises:

1. the addition of 900 µl of 0.5% ammonium molybdate in 0.8N HCl to each sample;
2. the addition of 100 µl of Fiske-Subbarow solution (0.025% 1-amino-2-naftol-4-sulfonic acid + 0.5% Na_2SO_3 + 15% Na_2S_2O5);
3. after incubation at room temperature for 30 min, the OD is measured at 700 nm;
4. the protein concentration is determined by the method of Lowry (Lowry et al., J. Biol. Chem. 193, 265-275, 1951)

d) The measurement of free phosphate using glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase in a linked enzyme assay according to Trentham et al. (Biochem. J. 126, 635-644, 1972).

4. Measurement of TPP activity using radioactive trehalose-6-phosphate as substrate

Alternatively TPP activity can be measured by direct measurement of radioactive phosphate using radioactive trehalose-6-phosphate in the extracts as has been described by Vuorio et al. (Eur. J. Biochem.216, 849-861,1993). The method comprises the

following steps:

a) cultures are grown to the desired density and are cooled quickly on ice;

b) preferably, at least 400 mg of cells are harvested by centrifugation for 3 min at 3000 rpm;

c) cells are washed twice with ice-cold distilled water;

d) cells are resuspended in 990 µl extraction buffer consisting of 968 µl of 50 mM Imidazole (Merck), 2 µl of 0.5 M EDTA (Tritriplex III Merck), 20 µl of 0.1 M $MgCl_2$ (UCB),

e) cells are transferred to screw capped tubes and an equivalent of 0.5 ml glass beads with 0.5 mm diameter are added;

f) just before extraction 10 µl of a 100 mM PMSFsolution (Sigma) in methanol; is added to the cell mixture ;

g) the extraction is performed in a Fastprep apparatus (BIO101) for 20 seconds at level 4, and is repeated three times with a cooling step on ice in between. Alternatively the extraction can be performed on a vortex apparatus in glass tubes;

h) extracts are centrifuged at 4°C for 20 min at 14000 rpm;

i) 200 µl of the supernatant is loaded on a small Sephadex G25 column, made in a blue tip containing a siliconized glass bead. The tip is filled completely with pre-equilibrated G25 Sephadex (50 mM Tricine (Sigma) buffer pH7). The tips are centrifuged once before application of the extract for 1 min at 800 rpm. The extract is centrifuged for 1 min at 1000 rpm;

j) 5 µl of the eluate is added to 45 µl assay solution consisting of 27.5 mM Tris-Cl (pH7.4), of 5.5 mM $MgCl_2$, 1 mg/ml BSA and 0.55 mM trehalose-6-phosphate (specific activity 854 cpm/nmol);

k) the assay mixture and the control mixture are incubated for 1 hour at 30°C;

l) the assay mixture is boiled for 5 min to stop the reaction;

m) AG1-X1 is added to the mixture and the mixture is incubated for 20 min at room temperature.

n) centrifugation of the micro-centrifuge tubes for 5 min at 14000 rpm

o) after centrifugation 400 µl of the supernatant is counted in a liquid scintillation counter.

SACCHAROMYCES CEREVISIAE STRAINS USED INCLUDING THE *TPS2Δ* MUTANT

The following *S. cerevisiae* strains were used in the experiments :

Name	Relevant genotype	Complete genotype
W303.1A	Wild-type	a *leu2-3,112 ura3-1 tpr1-1 his3-11,15 ade2-1 can1-100 GAL SUC2*
YSH448	*tps2Δ*	a *leu2-3,112 ura3-1 tpr1-1 his3-11,15 ade2-1 can1-100 GAL SUC2 tps2Δ::HIS3*
PVD32	W303.1A prototrophic	
PVD23	*tps2Δ* prototrophic	
PVD45	YEpTPS2	*a leu2-3/112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100 GAL SUC2 tps1Δ::TRP1 tps2Δ::LEU2 + pSAL4/TPS2 (URA3)*
YSH339	W303.1A alpha	alpha *leu2-3,112 ura3-1 tpr1-1 his3-11,15 ade2-1 can1-100 GAL SUC2*
PVD190	heterozygotic W303 diploid	*leu2-3,112 / LEU2 ura3-1/ URA3 trp1-1/ TRP1 his3-11,15/ HIS3 ade2-1/ ADE2 can1-100 GAL SUC2*
PVD191	*tps2Δ* /WT diploid	*leu2-3,112 ura3-1/ URA3 trp1-1/ TRP1 his3-11,15/ HIS3 ade2-1/ ADE2 can1-100 GAL SUC2 tps2Δ::LEU2*

The construction of the prototrophic *S. cerevisiae* strains was performed as follows:

1. <u>Construction of PVD32</u>.

W303.1A was first transformed (LiAc method according to Gietz et al (1992), Nucleic Acids Research **20**: 1425 – Gietz et al (1995), Yeast **11**: 355-60) with a plasmid containing the *HIS3* marker. Unless otherwise stated, all yeast transformation steps were performed in accordance with this method. The plasmid pJJ215 was linearized with *Nhe*1, which cuts in the marker. The complete plasmid will integrate at the location of the *his3* marker and as a result one wild-type *HIS3* marker is present in the genome. The resulting strain is PVD1: W303-1A *HIS3*.

Subsequently PVD1 was transformed with a plasmid containing the *URA3* marker. The plasmid YIplac 211 was linearized with *Eco*RV, which cuts the marker. The complete plasmid will integrate at the location of the *URA3* marker and as a result one wild-type *URA3* marker is present in the genome. The resulting strain is PVD2: W303-1A *HIS3 URA3*. PVD2 was transformed with a plasmid containing the *LEU2* marker. The plasmid YIplac128 was linearized with *Eco*RV, which cuts in the marker. The complete plasmid will integrate at the location of the *leu2* marker and as a result one wild-type *LEU2* marker is present in the genome. The resulting strain is PVD16: W303-1A *HIS3 URA3 LEU2*. PVD6 was transformed with a plasmid containing the *ADE2* marker. The plasmid pASZ10 was linearized with *Eco*RV, which cuts in the marker. The complete plasmid will integrate at the location of the *ade2* marker and as a result one wild-type *ADE2* marker is present in the genome. The resulting strain is PVD29: W303-1A *HIS3 URA3 LEU2 ADE2*. Finally, PVD29 was transformed with a plasmid containing the *TRP1* marker. The plasmid YIplac204 was linearized with *Eco*RV, which cuts in the marker. The complete plasmid will integrate at the location of the *trp1* marker and as a result one wild-type *TRP1* marker is present in the genome. The resulting strain is PVD32: W303-1A *HIS3 URA3 LEU2 ADE2 TRP1*.

2. Construction of PVD23

YSH448 was first transformed with a plasmid containing the *HIS3* marker. The plasmid pJJ215 was linearized with *Nhe*1, which cuts in the marker. The complete plasmid will integrate at the location of the *his3* marker, and as a result one wild-type *HIS3* marker is present in the genome. The resulting strain is PVD11: *tps2Δ::LEU2 HIS3*. PVD11 was subsequently transformed with a plasmid containing the *URA3* marker. The plasmid YIplac211 was linearized with *Eco*RV, which cuts in the marker. The complete plasmid will integrate at the location of the *URA3* marker and as a result one wild-type *URA3* marker is present in the genome. The resulting strain is PVD7: *tps2Δ:: LEU2 HIS3 URA3*. PVD7 was transformed with a plasmid containing the *TRP1* marker. The plasmid YIplac204 was linearized with *Eco*RV, which cuts in the marker. The complete plasmid will integrate at the location of the *trp1* marker and as a result one wild-type *TRP1* marker is present in the genome. The resulting strain is PVD18: *tps2Δ::LEU2 HIS3 URA3 TRP1*. Finally, PVD18 was transformed with a plasmid containing the *ADE2*

marker. The plasmid pASZ10 was linearized with *Eco*RV, which cuts in the marker. The complete plasmid will integrate at the location of the *ade2* marker and as a result one wild-type *ADE2* marker is present in the genome. The resulting strain is PVD23: *tps2Δ::LEU2 HIS3 URA3 TRP1 ADE2.*

3. Construction of PVD190.

Strain PVD190 is a diploid *S. cerevisiae* strain made by a cross between the W303 prototrophic strain (PVD32) and the W303 haploid auxotrophic strain (YSH339).

4. Construction of PVD191.

Strain PVD191 is a diploid strain made by a cross between the *tps2Δ* prototrophic strain (PVD32) and the W303 haploid auxotrophic strain (YSH339).

Methods for integrative transformation of yeast cells and other useful recommendations can be found in Ausubel et al (1999), "Short Protocols in Molecular Biology, 4th ed. John Wiley & Sons, New York", and unit 13.10 in particular.

CONSTRUCTION OF THE *CANDIDA ALBICANS* DOUBLE *TPS2Δ* KNOCK OUT MUTANT

The following *C. albicans* strains were used in the experiments :

Name	Relevant genotype	Complete genotype
SC5314	Wild-type	Clinical isolate
CAI4	*ura3Δ/ura3Δ*	*ura3Δ::imm434/ ura3Δ::imm434*
CC5	*TPS2/tps2Δ*	*ura3Δ::imm434/ ura3Δ::imm434* *TPS2/tps2Δ::URA3*
CC17	*tps2Δ/ tps2Δ*	*ura3Δ::imm434/ ura3Δ::imm434* *tps2Δ::URA3/ tps2Δ::URA3*

The construction of these *C. albicans* strains is described hereinbelow.

1. Genomic DNA isolation from yeast cells

All DNA procedures, including DNA isolation from *Candida albicans* yeast cells, are performed according to standard protocols (Sambrook et al. (1989) in "Molecular Cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, CSH, New York"; Ausubel et al (1994) in "Current Protocols in Molecular Biology, Current Protocols, USA. John Wiley & Sons, New York"; Ausubel et al (1999) in "Short Protocols in Molecular Biology, 4th ed. John Wiley & Sons, New York").

2. Isolation and cloning of the *Candida albicans TPS2* sequence (*CATPS2*)

A *Candida albicans* sequence homologous to the *Saccharomyces cerevisiae TPS2* gene was found in the *Candida albicans* database (http://www-sequence.stanford.edu/group/candida). This sequence, located in contig 4-3098, is further referred to as the *Candida albicans TPS2* gene, or *CaTPS2*. Both sequences were aligned for maximal amino acid similarities by making use of the CLUSTAL W (1.8) multiple sequence alignment software. Conserved regions of the amino acid sequences were aligned to give the best fit. Identical residues are indicated by an asterisk (*). Gaps in the amino acid sequence are represented by dots (--). A colon (:) stands for strong similarity, a dot (.) stands for weak similarity. The two putative phosphatase boxes are indicated in bold italic and are underlined.

The *CaTPS2* gene and its flanking sites were then isolated from a *Candida albicans* wild-type strain SC5314, using the following 30 and 31 bp oligonucleotide primers (Pharmacia) for PCR (Polymerase Chain Reaction) amplification:

CaTPS2FOR2: 5' GAGTCGACCTCACCTGAGGCATCCACATAC 3'

CaTPS2REV2: 5' GAGGTACCGTGTAATCCGGACATTAACTCCG 3'

whereby the forward primer (FOR2) contains the recognition site for the restriction enzyme *Sal*I and the reverse primer (REV2) that of *Kpn*I (see boxes).

PCR amplifications (30 cycles) and analysis were performed according to standard protocols (for references see above). PCR amplification with the designed primers (Pharmacia) yielded a fragment of 3171 bp long, which contains apart from the *TPS2* reading frame an additional 523 bp upstream and 639 bp downstream sequences.

3. Construction of the *C. albicans* disruption construct

Both the PCR fragment containing the *TPS2* sequence and the cloning plasmid pUC19 (New England Biolabs) were digested with *Sal*I and *Kpn*I, and after separation on a 1% agarose gel, subsequent slicing and elution, the PCR product was ligated into the cloning vector, to form the 6470 bp pUC19/*CaTPS2* with which *E. Coli* cells were then transformed via the $CaCl_2$ method (Ausubel et al (1999) in "Short Protocols in Molecular Biology, 4th ed. John Wiley & Sons, New York", unit 1.8). Alternative transformation methods are well known in the art. The DNA of one of the positive colonies was then digested with the restriction enzymes *Sna*BI and *Nsi*I, cutting just in the beginning and at the end of the *CaTPS2* open reading frame, leaving only 8 and 10 amino acids at the C- and N- terminus respectively. The large fragment (3885 bp) thus contains the vector plus the flanking sites of the *CaTPS2* gene. In this vector, the *HisG-URA3-HisG* cassette was cloned. The *URA3* blaster cassette, located on a fragment of 3948 bp, was isolated from plasmid pMB7-A (Fonzi W.A. and Irwin M.Y. (1993), Genetics **134**: 717-728) via digestion with *Bgl*II, Klenow fill in of the sticky ends and digestion with *Pst*I. This cloning strategy resulted in the 7384 bp pUC19/*Catps2Δ::HisGURA3HisG* vector. The final disruption construct (pUC19/*Catps2Δ::HisGURA3HisG*) is digested with *Avr*II and *Spe*I and the 4814 bp fragment containing the flanking sites of the *CaTPS2* gene and the *URA3* blaster cassette used to transform cells of CAI4 *Candida albicans URA3*⁻ strains (*Δura3::imm434/Δura3::imm434*). Once again, all DNA manipulations and procedures, like restriction, ligation, elution via gel electrophoresis etc., were performed according to standard protocols (for references see above).

4. Generation of a heterozygous *C. albicans TPS2/tps2Δ* strain

Competent CAI4 cells were prepared using a modified LiAc (Lithium acetate) method (Sanglard et al (1996), Antimicrobiol. Agents Chemother. **40**: 2300-2305) . Briefly, yeast cells are grown, harvested and pelleted as recommended. Cells are then resuspended in buffered lithium solution, freshly prepared. Next, 50 µl of this yeast cell suspension was added to 300 µl of PEG solution (PEG4000 of Merck), together with 50 µg of carrier DNA (sperm carrier DNA from Clontech, Yeastmaker carrier DNA Cat No. K1606-A) and 30 µg of the DNA fragment. After mixing, the cells were incubated at 30 °C for 1 hour in an incubator with shaker. Subsequently they were given a heat shock at 42 °C for 15 min. After a brief centrifugation step (about 15 sec), the supernatant was

removed and the cells were resuspended in 300 µl of 1x TE buffer. The cells were plated on SDglucose-ura containing plates and incubated at 30°C for 2 to 3 days. 14 transformants were obtained as such. Colonies were picked up after three days.

Alternative methods for introduction of DNA into yeast cells comprise spheroplast transformation and transformation by electroporation, methods well known by those skilled in the art. The standard LiAc method is described in Ausubel et al (1999) in "Short Protocols in Molecular Biology, 4th ed. John Wiley & Sons, New York", unit 13.7 and unit 13 in general)

PCR analysis with a Diag3 (3' diag) 19 bp oligonucleotide primer (Pharmacia), annealing to the flanking sites of the *CaTPS2* gene outside of the fragment that has been used for the disruption, and with DiagHIS4, a 18 bp oligonucleotide primer (Pharmacia) annealing to a nucleotide sequence in the *HisG* sequence was used to verify the deletion of one of the *TPS2* alleles.

Diag3: 5' CCTTCATCGCCTGACTGAT 3'

DiagHIS4: 5' GCGTAAGCGGGTGTTGTC 3'

The results were confirmed via Southern blotting . Two different fragments of 2874 (wild-type allele) and 3224 (disrupted allele) bp long respectively were visualized as such. Genomic DNA (see above) was prepared from all 14 transformants and after digestion with *Eco*RI, separated on a 1% agarose gel and blotted onto nylon membranes (Amersham Pharmacia biotech). The membrane was hybridised with a P^{32}-labelled 579 bp probe containing the 3' flanking site of *CaTPS2,* prepared by digesting pUC19/*CaTPS2* with *Hind*III and *Sna*BI. P^{32} labelling, via [α-^{32}P]dATP, was done by using the HighPrime DNA labelling kit (Boehringer, Roche Molecular biochemicals, Cat No. 1 585 584). The molecular weight marker is marker VII from Boehringer (Cat No. 1 209 264). All steps involved with Southern hybridization and hybridization conditions therefore were performed according to standard protocols (for references see above). Visualization was performed via a Phospho Imager from Fuji (BAS-1000; Software, PCBAS 2.0).

It will be readily understood that radiolabelled probes as well as nonisotopic probes can be used in Southern blotting and that detection methods will vary in accordance with the type of probes used. Biotin and digoxigenin are the nonisotopic labels that are used most frequently (Ausubel et al (1999) in "Short Protocols in

Molecular Biology, 4th ed. John Wiley & Sons, New York", and units 2.9A, 2.10, 3.18-19 in particular).

5. Generation and characterisation of a homozygous *C. albicans tps2Δ/tps2Δ* strain

To knockout the second allele, the heterozygous *TPS2/tps2* *URA3*$^+$ strain is first plated on 5-fluoroacetic acid (5-FOA) medium (FOA medium, Ausubel et al (1999) in "Short Protocols in Molecular Biology, 4th ed. John Wiley & Sons, New York", and units 13.1 and 13.10 in particular) to generate a *URA3*$^-$ strain. *URA3*$^+$ strains cannot survive on media containing the pyrimidine analog 5-FOA (5-FOA selection, modified method of Boeke et al (1984), Mol. Gen. Genet. 197: 345-346; uridine is used instead of uracil). Consequently, one can select for strains that have lost the *URA3* gene via homologous recombination. Loss of the *URA3* marker via homologous recombination is facilitated due to the presence of two identical *HisG* sequences flanking the *URA3* gene in the *URA3* blaster cassette.

The FOA$^+$ura$^-$ heterozygous clone, was transformed (LiAc method, see above) with the *TPS2* deletion construct (*URA3* disruption cassette) in order to obtain a homozygous deletion strain. After several rounds of transformation and checking by PCR , one transformant was found that did not show the band corresponding to the wild-type allele anymore in the PCR test. For this PCR analysis, the Diag3 (3' diag, see above) and Diag5 (5' diag) primers (Pharmacia) were used:

Diag5 : 5' ACCGTCGTGCTGATCCTG 3'.

The 18 bp oligonucleotide Diag5 primer is located in the open reading frame of the *TPS2* gene. For the PCR analysis with DNA isolated from 4 positive colonies, a mixture of the three following primers: Diag3, DiagHIS4 and Diag5 was used in the analysis. The molecular weight marker was the Smart ladder of Eurogentec (Cat No.: MW-1700-10). The presence of only a 1100 bp fragment indicated that in the corresponding transformant the two *TPS2* alleles were deleted. This was confirmed by Southern analysis, using the same P^{32}-labelled probe and marker, cutting the DNA with the same restriction enzymes as before. Once more, all steps involved with Southern hybridization and visualization of the results were performed according to standard protocols (for references see above). The upper bands in the blot are probably due to incomplete

digestion of the DNA. Further evidence for a double knock out was provided by repeating the Southern blot but using a P^{32}-labelled *TPS2* specific probe. The probe consisted of a P^{32}-labelled 548 bp *Nde*I-*Bam*HI fragment of the *CaTPS2* coding region. Labelling and hybridization were performed under the same conditions as defined above. The *TPS2* specific probe did not hybridise to any DNA fragment from the double deletion mutant.

EXPERIMENTAL

SACCHAROMYCES CEREVISIAE

Sensitivity of the S. cerevisiae *tps2Δ* strain towards antifungals

Sensitivity towards the antifungals itraconazole and ketoconazole was tested in microtiter wells using a bioscreen C apparatus (Life sciences, Labsystems) and on solid culture media.

S. cerevisiae cells were pre-grown in YPglucose medium till stationary phase. The cells were diluted to a initial optical density of 0.05 OD and 300 µl of cell suspension was added to each well of a microtiter plate. Three µl of the correct stock solution of the compound, dissolved in DMSO, were added to each well. For the control reactions, only DMSO was added. The microtiter plates were placed in the bioscreen C apparatus and were incubated at 33°C or 37°C, with medium intensity shaking (30 seconds shaking per minute). The optical density at 600 nm was measured every 30 min.

The experiments proved to be reproducible if the strains are pre-grown and collected at the stationary phase. During the exponential growth phase on glucose medium, no trehalose synthesis occurs and hence no trehalose-6-phosphate is accumulated and hence the antifungals have similar effects on the *tps2Δ* strain as on the wild-type (not shown).

At 37°C, the growth of the *tps2Δ* mutant strain PVD23 in the presence of 10^{-5} to 10^{-8} M Itraconazole or 10^{-5} to 10^{-8} M Ketoconazole was compared to the growth of strain PVD32 and to the growth of both strains in the absence of antifungal agents, as shown in Figure 2. At this temperature the *tps2Δ* strain is already affected by itself for growth on the glucose medium (YPD + DMSO).

The effect of the addition of 10^{-7} M Itraconazole and 10^{-6} Ketoconazole on the

growth curve of prototrophic *tps2Δ* strain PVD32 and wild-type strain PVD23 at 33°C is shown in Figures 3 and 4 respectively.

Addition of 10^{-7} M Itraconazole or 10^{-6} M ketoconazole to the *tps2Δ* strains had a dramatic effect on their growth, with no detectable growth occurring after 24 hours.

The effect of different concentrations of itraconazole on the growth at 33°C of the diploid wild-type strain (PVD190) and the diploid heterozygous *tps2Δ* strain PVD191 was tested and the results are presented in Figure 5.

The effect of Itraconazole on the growth of *Saccharomyces cerevisiae* wild-type (PVD32) and *tps2Δ* (PVD23) strains using YPD plates containing this compound was tested and the results are presented in Figure 6. Cells were pregrown on YPDglucose medium till stationary phase. The cells were diluted to an initial OD of 0.5. This corresponds to approximately 10^7 cells/ml. Starting from this initial cell suspension, 10-fold serial dilutions were made and from each dilution 10 µl suspension was spotted on YPD plates containing different concentrations of Itraconazole. The plates were incubated at 33°C and the occurrence of growth was estimated after 1 and 2 days.

At 33°C and 10^{-7} M Itraconazole incorporated in the medium, the *tps2Δ* strain was inhibited for growth, even after two days incubation, thus confirming the results obtained with the liquid media.

The *S. cerevisiae TPS2* deletion strain is sensitive to osmotic and heat stress.

The growth characteristics of wild-type (PVD32) and *tps2Δ* (PVD23) *S. cerevisiae* strains were tested in the presence of either 1.5 M sorbitol or 5% NaCl. The strains were pre-grown on YPD plates to stationary phase. The cells were diluted to an OD of 0.5. This corresponds to approximately 10^7 cells/ml Starting from this initial cell suspension, 10-fold serial dilutions were made and from each dilution 10 µl suspension was spotted on YPD plates containing either 1.5 M sorbitol or 5% NaCl. Plates of the different media were inoculated with 5 µl of 10-fold serial dilutions and incubated at 30 °C.

The effect of heat stress on the prototrophic wild-type and *tps2Δ* strains was tested by inoculating YPD plates with 10 µl of cell suspension and incubating the plates at 37°C, 39°C and 41°C. Visual readings were done after 24 and 48 hours.

Figure 7 shows the effect of the osmotic and heat stress on the growth of the

tested strains. The results clearly show that a *tps2Δ* strain is more sensitive to osmotic or salt stress in comparison with a wild-type strain.

A heat stress experiment has been repeated where the yeast cells were incubated at 39°C. A *tps2Δ* strain cannot grow at this temperature, whereas a wild-type strain grows perfectly well. Remarkable was the fact that the *tps2Δ* strain in the W303.1A background did not show any growth defect on plates at 37°C. In liquid media, however, there is a difference in growth rate between the wild-type and the mutant. At 41°C, none of the strains grew.

CANDIDA ALBICANS

A *Candida albicans* sequence homologous to the *Saccharomyces cerevisiae TPS2* gene (Candida albicans database, http://www-sequence.stanford.edu/group/candida) (Figure 8) was isolated from a *Candida albicans* wild-type strain SC5314. PCR amplification with the designed primers (REV2 and FOR2) yielded a fragment of 3171 bp long, which contains apart from the *TPS2* reading frame an additional 523 bp upstream and 639 bp downstream sequences. Figure 9 shows the genomic organisation of the *CaTPS2*gene and its flanking sites with indication of the relevant restriction sites and with indication of the two primers used to amplify the gene (REV2 and FOR2). The diag primers (3' diag and 5' diag) are diagnostic primers used to check for deletions in the strain.

The cloning strategy used to obtain a heterozygous *C. albicans* disruption construct, with which competent CAI4 cells were transformed (modified LiAc method according to Sanglard et al, see above) is summarized in Figure 10. PCR analysis with a Diag3 (3' diag) 19 bp oligonucleotide primer (Pharmacia), annealing to the flanking sites of the *CaTPS2* gene outside of the fragment that has been used for the disruption, and with DiagHIS4 (Pharmacia), a 18 bp oligonucleotide primer annealing to a nucleotide sequence in the *HisG* sequence was used to verify the deletion of one of the *TPS2* alleles. The results were confirmed via Southern blotting (Figure 11), showing two different fragments of 2874 (wild-type allele) and 3224 (disrupted allele) bp long respectively.

To knockout the second allele, the heterozygous *TPS2/tps2Δ URA3*$^{+}$ strain is first plated on 5-fluoroacetic acid (5-FOA) medium, to select subsequently for strains that have lost the *URA3* gene via homologous recombination (*URA3*$^{-}$ strains). Loss of the

URA3 marker via homologous recombination is facilitated due to the presence of two identical *HisG* sequences flanking the *URA3* gene in the *URA3* blaster cassette.

The FOA^+ura^- heterozygous clone, was then transformed with the *TPS2* deletion construct in order to obtain a homozygous deletion strain. After several rounds of transformation and checking by PCR , one transformant was found that did not show the band corresponding to the wild-type allele anymore in the PCR test. For this PCR analysis, the Diag3 (3' diag, see above) and Diag5 (5' diag) primers were used. Figure 12A shows the result of the PCR analysis on DNA isolated from 4 positive colonies. A mixture of the three following primers: Diag3, DiagHIS4 and Diag5 was used in the analysis. Wild-type alleles should give a fragment of 1544 bp, whereas deletion alleles should give a fragment of 1100 bp. The presence of only a 1100 bp fragment, indicates that in the corresponding transformant the two *TPS2* alleles are deleted (lane 2 in Figure 12A). This was confirmed by Southern analysis (lane 9 in Figure 12B). The upper bands in the blot are probably due to incomplete digestion of the DNA. Further evidence for a double knock out was provided by repeating the Southern blot but using a *TPS2* specific probe . This probe did not hybridise to any DNA fragment from the transformant corresponding to lane 9 (data not shown).

Candida albicans TPP activity (as measured via the method of Bencini, see above) as low as 6.9 nKat/g protein for the *tps2Δ/tps2Δ* compared to values of 231.3 for the wild-type (*TPS2/TPS2*) and 147.3 for the heterozygous knock out strain (*TPS2/ tps2Δ*), provided a third proof for the double knock out in *tps2Δ/tps2Δ*. Thus, whereas in the heterozygous strain there is still approximately half the TPP activity of that found in the wild-type strain, in the putative double deletion strain there is no TPP activity at all any more. Because of this reason, the cells are no longer able to convert trehalose-6-phosphate into trehalose under conditions where normal cells would accumulate large quantities of trehalose. It is assumed that accumulation of large quantities of trehalose-6-phosphate under these conditions is highly toxic to the cells. Trehalose-6-phosphate is a strong acid and will therefore cause intracellular acidification, which will negatively influence cellular metabolism or growth or even inhibit growth of double deletion yeast strains. Hyperaccumulation of trehalose-6-phosphate also sequestrates free orthophosphate (Pi) and in this way will negatively influence glycolytic flux and energy (ATP) generation. As such, total cellular energy metabolism can be severally disturbed.

Growth curves were established for *Candida albicans* cells grown in microtiter plates in YPglucose (A) and YPgalactose (B) medium. Plates were incubated in a bioscreen C apparatus (Life sciences, Labsystems) at temperatures of 41°C, resulting in the accumulation of trehalose in yeasts at least in wild-type cells. The *C. albicans* strains tested in accordance with the particular embodiment were the *TPS2/TPS2* SC5314 wild-type strain, the heterozygous *TPS2/tps2*Δ strain (CC5) and the homozygous *tps2Δ/tps2Δ* strain (CC17). Cell lines of these three strains were deposited with the BCCM-IHEM (Belgian Co-ordinated Collections of Microorganisms, Scientific Institute of Public health, Louis Pasteur Mycology (IHEM), J. Wytsmanstraat 14, B-1050 Brussels, Belgium) with the accession numbers IHEM 17208 (SC5314) IHEM 17210 (CC5) and IHEM 17209 (CC17) on 24 July, 2000.

The growth curves at 41°C already showed that there is a clear extension of the lag phase and a clear inhibitory effect on the growth rate when respectively one or two of the alleles of the *TPS2* gene are deleted and especially when the two *TPS2* alleles are deleted. Such growth curves provide proof of principle for the fact that as a result of *TPS2* knock out, accumulation of trehalose-6-phosphate in cells, cytotoxic to at least yeast cells in higher concentrations, can hamper or even inhibit cell growth.
At 43°C, the effect was even more pronounced. At this particular temperature, the wild-type *Candida albicans* still grew quite well, whereas heterozygous and especially homozygous deletion of *TPS2* resulted in a dramatic drop in growth rate, presumably as a result of accumulation of trehalose-6-phosphate in the cells. This was observed both for galactose (YPgalactose) and glucose (YPglucose) medium (Figures 13 A and B).

SCREENING ASSAY

Several known inhibitors of different phosphatase enzymes have been tested, such as vanadate, tetramisole, (-)-p-bromotetramisole, (+)-p-bromotetramisole, levamisole, N-ethylmaleimide (NEM) and Dithiodinitrobenzoate (DTNB) for their inhibitory effect on TPP activity.

To perform a screening assay with the different candidate inhibitory compounds, *Saccharomyces cerevisiae* strain PVD45 (PVD45: a *leu2-3/112ura3-1 trp1-1 his3-11/15 ade2-1 can1-100GAL SUC2 tps1Δ::TRP1 tps2Δ::LEU2 + pSAL4/TP2S (URA3))* was used which over-expresses *TPS2* from *S. cerevisiae*.

The screening assay comprises the following steps:

1. Preparation of the extracts.

The *S. cerevisiae* strain PVD45 was grown in 50 ml SDgal-ura medium to stationary phase. Extracts were prepared according to the protocol described previously (see preparation of the extracts).

2. Screening of the candidate inhibitory compounds NEM and DTNB.

Stock solutions of 100 mM N-ethylmaleimide (NEM) and 100 mM Dithiodinitrobenzoate (DTNB) are prepared in water and ethanol respectively. From these stock solutions, serial dilutions of 0.1 mM, 1mM and 10 mM, are made. For the experiments 56 µl of each dilution of the compound is added to the Assay I solution consisting of 40 µl of 200 mM Tricine buffer (pH7), 20 µl of 0.1 M $MgCl_2$, 20 µl trehalose-6-phosphate (Sigma) and 68 µl H_2O (mix1, see above). For the Assay I control mixture 20 µl trehalose-6-phosphate is omitted and replaced by 20 µl H_2O. Thus in the experiments the following final concentrations of the tested compounds NEM and DTNB were used: 35 mM, 3.5 mM, 0.35 mM, 35 µM and 0 µM (= control).

To the assay mixture 20 µl of extract is added.

The assay mixtures and the control mixtures are incubated for 30 min at 30°C; subsequently boiled for 5 min to stop the reaction and cooled down to room temperature. The micro-centrifuge tubes were centrifuged for 5 min at 14000 rpm.

Measurement of the trehalose-6-phosphate activity is performed according to the methods described previously. The chemical test, based on the method of Bencini (1983), might be preferred over the enzymatic test (EnzChek ™) since it is linear over a broad range of concentrations and is less prone to interference and the generation of false positives compared to the enzymatic test.

The marked inhibition of trehalose-6-phosphate phosphatase activity in *S. cerevisiae* strain PVD45 by NEM and DTNB is shown in Figures 14 and 15. Bencini's method for the measurement of free orthophosphate, clearly demonstrated an inhibitory effect of NEM and DTNB at 10^{-4} and 10^{-5} M, whereas at these concentrations none of

the following compounds inhibited TPP activity: vanadate, tetramisole, (-)-p-bromotetramisole, (+)-p-bromotetramisole, levamisole, chlorogenic acid. The above mentioned selective and specific inhibitory action of NEM and DTNB whereas general phosphatase inhibitors had no inhibitory effect on trehalose-6-phosphate phosphatase were the first indication that specific inhibitors can be found for TPP activity which will not interfere with phosphatase enzymes of host cells.

Assays as described above for a limited set of test compounds, were performed in micro-centrifuge tubes. An assay in accordance with the present invention further involves screening test inhibitory compounds from large libraries of synthetic or natural compounds. Synthetic compound libraries are commercially available from, for example, Maybridge Chemical Co. (Trevillet, Cornwall, UK), Comgenex (Princeton, NJ), Brandon Associates (Merrimack, NH), and Microsource (New Milford, CT), Chembridge Corporation (San Diego, CA). A rare chemical library is available from Aldrich Chemical Company, Inc. (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available from, for example, New Chemical Entities, Pan Laboratories, Bothell, WA or MycoSearch (NC), Chiron, or are readily producible. Plant extracts may also be obtained from the University of Ghent, Belgium. Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means. Performing the screening assays in microtiter plates, for instance 96-well microtiter plates, allows screening by an automated robotic system and as such the testing of large numbers of test samples within a reasonable short time frame. The above list of commercial libraries is non-exhaustive.

In addition, preferably the TPP inhibitors will not interfere with any essential metabolic process or pathway of the human, animal or plant in need of treatment. In the context of the present invention, a 5,000 compounds-collection (DIVERSet™) from Chembridge Corporation (San Diego, CA) was screened for identifying novel TPP-specific intracellular inhibitors in accordance with the present invention. DIVERSet™ is a unique set of drug-like, hand-synthesized small molecules, rationally preselected to form a "universal" library that covers the maximum pharmacophore diversity with the minimum number of compounds. As appreciated by those skilled in the art, other libraries such as those previously mentioned in the non-exhaustive list above, may be screened. Such screening may yield inhibitors, other than the ones explicitly disclosed in

the present invention but falling within the scope of the present invention, inclusive further screening with structure analogs.

Details on the large scale screening assay

DIVERSet™ compounds are delivered in 96-well microtiter plates each containing 80 compounds, with each compound representing about 0.1 mg of lyophilized material. In the context of the present invention, the compounds were dissolved in 33 µl of DMSO resulting in a mean concentration of 10^{-2} M. From these stock plates (Figure 17), 10^{-3} M working plates were then prepared via a 10-fold dilution in DMSO (total volume per well is 50 µl) (Working plates). Next, 2 µl of each well of the working plates was transferred to the corresponding well of a fresh microtiter plate, to which 148 µl of mix1 (see above) was added. For the controls, the 2 µl of test compound at 10^{-3} M were replaced by 2 µl pure DMSO (negative control) or 2 µl of a 10^{-3} M DTNB solution in DMSO (positive control) respectively. To each well 10 µl of a yeast extract with final protein concentration of about 10 mg/ml (for the large scale extraction procedure and measurement of total protein content see above) was then added, whereafter the plates were incubated for 30 minutes at 30°C in a standard incubator in the dark. Next, 15 µl of each well was transferred to the corresponding well of a fresh microtiter plate, to which 148 µl of the aqueous mixture at pH 5 of ammonium molybdate (100 mM) and Zinc acetate mixture (15 mM) and 37 µl of a 10% aqueous ascorbic acid solution (pH5) (see above) were added. Well A to F of the first column of each microtiter plate contained an orthophosphate (Pi) standard series (0, 0.02, 0.1, 0.2, 1 and 5mM in H_2O). 15 µl of the Pi standard series hereby replaced the otherwise 15 µl of test compound plus mix1 plus yeast extract. Screening of the test plates was performed after 20 minutes of incubation at 30°C in the Biomek robotic system , by measuring the optical density at 750 nm (OD_{750}) using a SPECTRAMAX spectrophotometer. Potential TPP inhibitors were in a second round of screening retested at 4 differential concentrations of 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M in DMSO respectively.

The strain that was used to prepare the extracts for the large-scale screening assay in accordance with this particular embodiment of the present invention, is a *Saccharomyces cerevisiae* wild-type strain (W303.1 A) in which the yeast *TPS2* gene is overexpressed. FPLC fractions (750 µl fractions of a superdex200 column (Amersham Pharmacia biotech)) containing TPP activity (fractions 11-13) were pooled, concentrated

on VIVASPIN columns (VIVAscience) by a 30 min centrifugation step at 3500 rpm and used for screening in the assay. The final protein concentration of the extract used for screening was about 10 mg/ml.

The screening assay for determination of inhibitors of TPP activity in microtiter plates is adaptable for automation and as such allows high throughput screening. In the framework of the present invention, the Biomek robotic system was applied. It will be understood by a person known in the art that equivalent automated screening methods could be used as well.

The first round of screening resulted in 86 compounds with TPP inhibitory actions similar to or better than that of 10^{-5} M DTNB ("good" TPP inhibition). These compounds with their respective numbers/positions in the DIVERSet™ compound library are shown in Table 1 (for their structures, see Figure 17). Subsequently, these 86 compounds were tested again, but this time at concentrations of 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M respectively in DMSO. As such, 5 compounds with good TPP inhibition activity could be identified. These compounds (113596, 113610, 133207, 136794 and 143067) are indicated in bold italic and marked with an asterisk in Table 1.

Table 1: Indication of number and position (plate number – position in the plate) of compounds identified in the DIVERSet™ library as being compounds with good TPP inhibition activity

Compound Number	**Position**	**Compound Number**	**Position**
♦100764	***♦PL 2 – D 4***	155043	PL 41 – G 8
112710	PL 11 – H 4	155137	PL 41 – G 10
114854	PL 13 – A 10	153298	PL 42 – G 4
115308	PL 13 – D 7	156323	PL 44 – A 11
115800	PL 14 – A 2	158594	PL 44 – B 6
115806	PL 14 – A 5	159194	PL 46 – A 7
117003	PL 14 – F 6	159195	PL 46 – B 7
117033	PL 14 – G 6	159189	PL 46 – C 6
121911	PL 16 – H 2	156457	PL 46 – D 5

**136794*	**PL 21 – H 5*	156643	PL 46 – G 4
**143067*	**PL 23 – A 9*	159193	PL 46 – G 6
150050	PL 23 – H 2	157408	PL 46 – H 7
102330	PL 25 – A 3	140894	PL 46 – H 8
102341	PL 25 – A 7	155270	PL 46 – H 10
105299	PL 27 – E 9	156945	PL 47 – B 4
105463	PL 27 – G 10	106160	PL 47 – B 7
111087	PL 30 – A 2	122621	PL 47 – C 11
111189	PL 30 – A 8	155044	PL 47 – F 4
115781	PL 31 – A 8	159239	PL 47 – G 5
116321	PL 31 – B 7	161339	PL 47 – G 8
128067	PL 33 – G 8	105553	PL 47 – H 7
135235	PL 34 – G 10	108489	PL 47 – H 10
142159	PL 35 – A 5	104499	PL 48 – G 5
144152	PL 35 – B 5	169111	PL 49 – H 8
141531	PL 35 – C 3	182557	PL 51 – F 6
143530	PL 35 – C 5	◆**113596*	◆**PL52-D3*
141882	PL 35 – C 10	◆**133207*	◆**PL 52 – E 10*
143736	PL 35 – D 5	216645	PL 54 – H 4
136265	PL 35 – D 6	218940	PL 56 – A 2
142389	PL 35 – D 7	217973	PL 57 – H 4
136286	PL 35 – G 3	147933	PL 60 – A 4
141951	PL 35 – H 9	152078	PL 60 – E 9
143463	PL 36 – D 6	215222	PL 61 – C 10
143092	PL 36 – F 6	217342	PL 61 – F 10
143462	PL 36 – H 6	133445	PL 62 – E 7
104966	PL 38 – B 5	117172	PL 62 – F 5
118196	PL 38 – G 3	◆*133805*	◆*PL 62 – G 7*
107036	PL 38 – G 8	◆**113610*	◆**PL 63 – A 8*
150049	PL 41 – D 6	113222	PL 63 – B 3
153960	PL 41 – D 8	113233	PL 63 – C 3

145704	PL 41 – E 7	175327	PL 63 – F 6
146000	PL 41 – F 7	151234	PL 63 – H 3
151547	PL 41 – G 3		
146002	PL 41 – G 7		

DIVERSet™ compounds 136794 and 143067 (for their structures, see Figure 17), were applied in 7 different concentrations (0, $1x10^{-7}$, $3x10^{-7}$, $1x10^{-6}$, $3x10^{-6}$, $1x10^{-5}$ and $3x10^{-5}$ M in DMSO respectively) to *S. cerevisiae* and from their inhibitory activity the IC_{50} calculated. Three DIVERSet™ compounds without any TPP inhibitory activity (109146, 116321 and 145704) and DNTB were included as respectively negative and positive controls (Figure 18). DIVERSet™ compound 136794, comparable in its TPP inhibiting action to DNTB at 10^{-5} M, is less active at lower concentrations. The calculated IC50 for this compound is $1.5x10^{-5}$ M. DIVERSet™ compound 143067, has a calculated IC_{50} of $3.1x10^{-7}$M under the given test conditions and in accordance with this particular embodiment of the present invention. Compound 143067 has the strongest TPP inhibitory potential at 10^{-7} M. The drop in activity at higher concentrations is most probably due to a bad solubility of this compound at high concentrations .

The inability to penetrate the cell (non-permeability), rapid degradation of a compound or a conversion to inactive forms once inside the cell are possible reasons for a compound to be non-active *in vivo*. Therefore, next to the *in vitro* tests described above, the activity of some of the selected compounds on the growth of the yeast *Saccharomyces cerevisiae* wild-type strains (*in vivo* screening) and was tested using the bioscreen C apparatus (Life sciences, Labsystems). This system allows to follow the effect of the compounds on up to 200 different cultures simultaneously. In Figures 19 A and B, the results are given for the effect of DIVERSet™ compounds 136794 and 143067 on the growth of a wild-type *S. cerevisiae* strain (W303.1A) grown in YPD (YPglucose) at 37°C, a temperature at which the yeast accumulates trehalose through the action of TPS and TPP enzymes. Other incubation conditions in the bioscreen C apparatus were as described above. The *in vivo* results confirmed the *in vitro* data and even the concentrations at which the DIVERSet™ compounds 136794 and 143067 are most inhibitory to TPP.

The 86 DIVERSet™ compounds with good TPP inhibitory activity, identified via

the screening method of the present invention (see above) were also tested on *Candida albicans* cells (whole-cell assay). *In vivo* inhibition tests with the *TPS2/TPS2 C. albicans* wild-type strain (SC5314), grown on YPglucose medium at 43°C, identified DIVERSet™ compounds 133207, 133805 and 113610 (indicated in bold italic and marked by a diamond in Table 1) as the strongest inhibitors under the given test conditions (10^{-5} and 10^{-7}M in DMSO). Control compounds were DTNB and NEM (positive controls) and DMSO (negative control). The results are summarized in Figures 20 A and B.

To check for specificity, the inhibitory effect of the 3 DIVERSet™ compounds 133207, 133805 and 113610 was tested on the *C. albicans tps2Δ/tps2Δ* strain. This strain was grown at 39°C instead of at 43°C, since at the latter temperature the strain is not growing so well (see above). Since the Tps2 (TPP) enzyme is absent in the *tps2Δ/tps2Δ* strain (double deletion mutant), one should not see an inhibitory effect or only a minor effect of the compounds, as demonstrated in Figures 21 A and B. Test compounds were added at concentration of 10^{-5} (A) and 10^{-7}M (B) in DMSO. Controls were the same as for the wild-type strain.

The 3 aforementioned compounds proved to be specific inhibitors of TPP as the compounds do not or only slightly affect the growth of the double deletion mutant. DIVERSet™ compounds nr 133207 and 113610 have a strong effect on the growth rate of the wild-type *Candida albicans* strain, whereas compound 133805 is less effective for growth inhibition with wild-type *Candida albicans*. Remarkably, in the presence of the latter compound the cells grow a little slower in the beginning but after some hours cells stop growing completely. Since there was no effect at all of this compound in the *tps2Δ/tps2Δ* background, this might indicate that it also acts on Tps2 (TPP). In addition to compounds nr 133207 and 113610 there was a third DIVERSet™ compound with a strong inhibitory effect on the growth rate of *Candida* cells, namely compound nr 113596. The presence of DTNB did not seem to have an effect on the growth of wild-type *Candida* cells under the given conditions. Addition of NEM caused a slight reduction in the growth rate. The inhibitors identified in accordance with the present invention are thus superior in their TPP-specific inhibitory activity to NEM and DTNB at 10^{-5}M, especially *in vivo*.

The two compounds causing strong growth inhibition in *Saccharomyces*

cerevisiae, DIVERSet™ compounds 136794 and 143067, were also tested on *Candida albicans*. The compounds were tested at 10^{-7} M only, since at 10^{-5} M compound 143067 was less inhibitory in *Saccharomyces cerevisiae* (see above). Also in *Candida albicans* this compound caused significant growth inhibition at 10^{-7}M in DMSO. The inhibition is as strong as with DIVERSet™ compound 113610 (positive control). Another compound, with comparable activity was identified, namely compound nr 100764 (for structure, see Figure 17). Figure 22 shows the compound's behavior compared to compounds nr 136794, 143067 and 113610 (positive controls) and DMSO (negative control). Growth curves were established for a wild-type *C. albicans* strain (SC5314), grown at 43°C on YPD medium in the presence of 10^{-7} M of the test compounds.

IN VIVO TESTING OF THE POTENTIAL OF *CANDIDA TPS2* AS A POTENTIAL TARGET FOR ANTIFUNGALS.

Three strains of *C. albicans*, SC5314 (WT, deposit IHEM 17208)), CC5 (*TPS2/tps2* , deposit IHEM 17210) and CC17 (*tps2 /tps2* , IHEM 17209) were injected intravenously with 10^6 and 10^7 cells in mice (Balb/C). The results are presented in Fig. 23A (10^6) and 23B (10^7).

When mice were injected with 10^7 cells of wild type *C. albicans*, the mean survival time was about two days. After three days all mice were dead. After injection with the *tps2 /tps2* strain the mean survival time was about 4 days. After 5 days all mice were dead. The heterozygous deletion strain behaved in an intermediate way. When the mice were injected with 10^6 cells, those injected with wild type *C. albicans* were all dead after 13 days. The mice that were injected with the heterozygous mutant survived for 40 % this treatment (up to 40 days). Those injected with the homozygous mutant §(10^6 *tps2 /tps2 Candida albicans*) survived for 60% this treatment.

The organ burden of *C. albicans* (log10 c.f.u. per kidney) was comparable for all administered infective doses (Table 2).

The homozygous mice that survived the experiment were killed and the kidneys were investigated. No *Candida albicans* cells could be detected in these kidneys. This means that mice can completely recover from injection with 10^6 *tps2 /tps2 Candida albicans* cells, but not from 10^6 wild type *Candida albicans* cells. The mice injected with 10^6 *tps2 /tps2 Candida albicans* cells which died were examined. The kidneys of the

dead mice contained a virulent *Candida* strain not used in these tests, indicating that the cause of death was not caused by the injected cells.

Table 2

Challenge dose	Strain	Main genotype	Fungal burden (CFU)
1×10^7	SC5314	**TPS2/TPS2**	1.5×10^6
	CC5	**TPS2/tps2**	0.5×10^6
	CC17	*Tps2 /tps2*	1.3×10^6
1×10^6	SC5314	**TPS2/TPS2**	0.21×10^6
	CC5	**TPS2/tps2**	0.27×10^6
	CC17	*Tps2 /tps2*	3.9×10^6

Thus, an inhibitor of the *tps2* gene or of the TPP enzyme in accordance with the present invention which achieves the same inhibition as the heterozygous mutation (about 50%) may render *Candida albicans* non-lethal even in high injected doses.

FUNGICIDAL EFFECT OF THE *TPS2* MU T A TION IN *CANDIDA ALBICANS* UNDER STRONG STRESS CONDITION AT HIGH TEMPERATURE.

The *Candida albicans tps2* mutants were incubated under the strong stress condition of high temperature to investigate whether accumulation of trehalose-6-P only arrests cell proliferation or whether it causes cell death.

Cells of the wild type, *TPS2/tps2* and *tps2 /tps2* str a ins were incubated for different periods of time at 44 °C and aliquots were spotted in serial dilutions on YPD plates. Growth was scored after three days.

The results shown in Fig. 24 indicate that incubation at 44 °C for 7 h causes complete cell death in the homozygous *tps2 /tps2* str a in, whereas the survival of the wild type and heterozygous *TPS2/tps2* strain is comparable. This result shows that complete elimination of TPP activity by a strong inhibitor would be lethal under the stress condition of incubation at 44 °C or an equivalent stress condition induced by

osmotic pressure, immunological reactions or a stress raising compound.

TREHALOSE AND TREHALOSE-6-P OF WILD TYPE, *TPS2/TPS2* AND *TPS2/TPS2* *CANDIDA ALBICANS* CELLS UPON SHIFT TO HIGHER TEMPERATURES.

The aim of these experiments is to check whether also in *Candida albicans* trehalose accumulation occurs under stress conditions in the wild type strain and trehalose-6-P accumulation in the *tps2 /tps2* strain. In addition, information on how much trehalose would still be accumulated in the heterozygous *TPS2/tps2* strain was investigated.

1. Experimental setup.

Wild type (SC5314), *TPS2/tps2* (CC5) and *tps2 /tps2* (CC17) strains were grown overnight in YPD at 30 °C. In the morning the cells were washed and resuspended in 400 ml YPD. After three hours, the cultures were divided and 100 ml of each culture was further incubated at either 30 °C, 37 °C, 40°C or 43 °C in the case of trehalose and 37 °C or 43 °C in the case of trehalose-6-P. At time zero and at various time points after the shift, samples were taken for trehalose or trehalose-6-P determination.

2. Trehalose determinations

3 ml samples of each strain were taken at the different time points after the shift to the 4 different temperatures. The cells are filtered on a membrane filter (Gelman Sciences membrane filters # 60173) on a vacuum flask and washed with icecold water. The yeast cells are scraped off by a spatula and the wet weight is determined. The cells are extracted by boiling in 1 ml of 0.25M Na_2CO_3 for 20 min. After centrifugation, 10 µl of the supernatant is used for the trehalose determination. A standard curve using 0 to 5 mM trehalose is performed in parallel. The extracts are neutralized by adding 5 µl of 1M acetic acid. After neutralizing, 5 µl of the trehalase buffer (300 mM NaAc, 30 mM $CaCl_2$, pH 5.5) is added together with 20 µl of trehalase (purified from *Humicola grisea* var. thermoidea). The samples are incubated at 40 °C for 45 min. The glucose that is formed during this incubation is determined by the glucose oxidase/peroxidase reaction using Trinder reagent (Sigma). The amount of trehalose in the samples is determined based on the standard curve and the dilution. The results are shown in Fig. 25.

The wild type and the heterozygous deletion strain show a rapid increase in trehalose levels after the shift from 30°C to 40°C or 43 °C. There is also more trehalose (up to

three times more) in the *tps2 /tps2* strain. This indicates that there may be other probably aspecific phosphatases that are able to dephosphorylate to some extent the trehalose-6-P.

3. Trehalose-6-P (Tre6P)

The samples for trehalose-6-P (Tre6P) determination were taken as described by De Koning and Van Dam (Anal. Biochem. 204, 118-123, 1992). Tre6P was determined in the same extracts prepared for glycolytic metabolite determination. For Tre6P determination glucose was first removed by anion exchange chromatography and then the Tre6P was hydrolysed to glucose and Glu6P by phosphotrehalase (Bacillus subtilis) treatment. The glucose produced in this reaction was measured with glucose oxidase/ peroxidase and ortho-dianisidine. Using the total amount of protein in the sample as determined according to Lowry et al. (J. Biol. Chem. 193, 265-275, 1951) and the assumption of a yeast cytosolic volume of 12 µl per mg protein, cytosolic concentrations in mM were calculated. Fig. 26 shows the results of this experiment.

There is a two-fold increase in Tre6P levels in the heterozygous *TPS2/tps2* strain compared to the wild type (at 43 °C). In the *tps2 /tps2* strain, however, there is a more than 30 fold increase in the Tre6P level. The same fold induction is seen at 37 °C. The *tps2 /tps2* strain can not survive a heat treatment at 44 °C. Under these conditions there is a very large increase in Tre6P. This indicates that the high levels of Tre6P accumulated (hyperaccumulation) under the strong stress condition at high temperature cause the *tps2 /tps2* cells to die at this temperature.

It has been investigated whether the *tps2* mutation can enhance the growth inhibitory effect of known antifungals. Experiments were performed in a bioscreen apparatus with the three strains at three different temperatures (37 °C, 40 °C and 43 °C) in the presence of different concentrations of known antifungal compounds. All the azoles tested behaved in the same way. Figs. 27, 28, 29 show results of typical experiments with two concentrations of miconazole (left and right panel respectively) at three different temperatures (37, 40, 43°C).

The addition of antifungal compounds at concentrations higher than 10^{-7}M to *Candida albicans* cells results in a drop in growth rate. At lower concentrations and without stress, the compounds do not have any effect on the wild type strain. In the double knock-out *tps2 /tps2* strain, however, there is still a dramatic effect on growth

rate at 10^{-7}M and this inhibition is still visible at 10^{-8}M. Under stress conditions, the effect of antifungals (e.g. Micanozole) on the growth of the *tps2 /tps2* strain is even more pronounced. This indicates the usefulness of TPP inhibition for combined antifungal therapy with inhibitors of TPP together with known antifungal drugs and/or other compounds that induce or enhance the stress response of the cells.

The present invention further includes a method for treating a parasitic infection such as a fungal infection in a patient in need of such treatment, comprising administering to said patient an antiparasitic agent comprising an inhibitor as identified above, or determined according to one of above assay methods or a pharmaceutically acceptable salt, ester or pro-drug thereof. In additional at least a stress raising factor for the parasite may be co-administered.

More generally, the invention includes a method for treating a fungal, a bacterial or a protozoal infection, or a nematode, an insect, worm or mite infestation, in a human, animal or a plant in need of such treatment which comprises administering a specific inhibitor which inhibits the proper functioning of a cell enzyme of the parasite which converts with a low or high degree of specificity a sugar phosphate into a sugar or a sugar alcohol phosphate into a sugar alcohol that are accumulated in large quantities by cells for instance, but not exclusively, under conditions deviating from the optimal growth condition or as a reaction to stress conditions, the inhibition being either directly of the enzyme or indirectly, e.g. by suppressing the expression of the corresponding gene. The inhibitor may be determined by the assays described above.

Compositions in accordance with the present invention include a biologically or therapeutically effective amount of an inhibitory agent (either biocide or pharmaceutical) determined in accordance with a screening assay in accordance with the present invention. Therapeutically or biologically effective amounts are those quantities of the active agent of the present invention that afford prophylactic protection against the relevant infections or infestations in humans, animals or plants, and which result in amelioration or cure of an existing infection or infestation in humans, animals or plants.

The biologically or therapeutically active agents or compositions can be formed into dosage unit forms, such as for example, creams, ointments, lotions, powders, liquids, tablets, capsules, suppositories, sprays, or the like. For example, if the antifungal composition is formulated into a dosage unit form, the dosage unit form may contain an antifungal effective amount of active agent.

The active agents and compositions of the present invention are useful for preventing or treating parasitic, especially fungal infections in humans, animals or plants. Parasitic infection prevention methods in accordance with the present invention incorporate a prophylactically effective amount of an antiparasitic agent or composition. A prophylactically effective amount is an amount effective to prevent parasitic infection and will depend upon the parasite, e.g. fungus, the agent and the host. These amounts can be determined experimentally by methods known in the art. Parasite infection treatment methods incorporate a therapeutically effective amount of an antiparasitic agent or composition. A therapeutically effective amount is an amount sufficient to stabilize or to ameliorate a parasitic infection.

The prophylactically and/or therapeutically effective amounts can be administered in one administration or over repeated administrations. Therapeutic administration can be followed by prophylactic administration, once the initial parasitic infection has been resolved.

The parasitic, e.g. fungal agents and compositions can be applied to plants topically or non-topically, i.e., systemically. Topical application is preferably by spraying onto the plant. Systemic administration is preferably by application to the soil and subsequent absorption by the roots of the plant.

The antiparasitic agents in accordance with the present invention, e.g. antifungal agents and compositions, can be administered to animals topically or systemically.

Although the invention is described in detail with reference to specific embodiments thereof, it will be understood that variations which are functionally equivalent are within the scope of this invention. Such modifications are intended to fall within the scope of the appended claims. It will be understood that structural analogs of the compounds or substances disclosed in the present invention fall under the scope of it.

Various publications are cited herein, the disclosure of which are incorporated by reference in their entireties.

CLAIMS

1. A test method for assessing the activity of candidate substances as inhibitors of a first cell enzyme converting a sugar phosphate into a sugar or a sugar alcohol phosphate into a sugar alcohol in cells, the sugar or sugar alcohol being accumulated in large quantities by the cells for instance, but not exclusively, under conditions deviating from the optimal growth condition of the target cells or as a reaction to stress conditions, the inhibition being either directly of the first enzyme or indirectly; the method comprising the steps of:
Step 1: contacting a candidate inhibitor with a biological medium comprising the sugar phosphate or sugar alcohol phosphate and the first enzyme;
Step 2: measuring activity in the medium which depends upon the activity of the first enzyme;
Step 3: repeating steps one and two with further candidate inhibitors; and
Step 4: selecting at least one candidate inhibitor which reduces activity of the enzyme compared with the same medium without the inhibitor under the same conditions.

2. The test method of claim 1, wherein the enzyme inhibition slows down or impairs specifically the growth of the target organism, at least under stress conditions.

3. The method according to claims 1 or 2, wherein the first cell enzyme is a phosphatase which synthesizes a sugar or sugar alcohol as a reaction to stress.

4. The method according to claims 1 to 3, wherein the reduction in activity is preferably at least 25%, more preferably at least 50%, more preferably at least 75%, more preferably at least 85% and most preferably at least 95%.

5. The method according to any previous claim, further comprising the steps of:
step 5: assessing the activity of a second cell enzyme which is involved in the synthesis of the corresponding sugar phosphate or sugar alcohol phosphate; and the selecting step includes selection of inhibitors which reduce the activity of the first enzyme while maintaining a viable activity of the second enzyme.

6. The method according to claim 5, wherein a viable activity of the second enzyme is at

least 25%, more preferably at least 50% and most preferably at least 75% of the activity of the second enzyme in the same medium under the same conditions but without the inhibitor.

7. The method according to any previous claim, wherein the medium includes sub-cellular organelles or sub-cellular non-organelle components, a cell culture, or animal tissue, or an animal.

8. The method according to claim 7, wherein the sub-cellular organelles or sub-cellular non-organelle components or the cell culture are obtained from cells from an insect or a nematode or a fungus or a bacterium or a protozoa or a worm or a mite or any other organism expressing the first enzyme of any of the previous claims.

9. The method according to claim 7, wherein the medium is a cell culture and the first enzyme is an intracellular enzyme.

10. The method according to any previous claim wherein the first enzyme is an enzyme controlling a metabolic pathway which has an intermediary compound which is normally produced as a reaction to stress conditions and which is toxic to the cell.

11. The method according to claim 10, wherein the first enzyme is one of trehalose-6-phosphatase, glycerol-3-phosphatase, mannitol-1-phosphatase, sorbitol-6-phosphatase, arabitol-5-phosphatase, and erythritol-4-phosphatase.

12. The method according to any previous claim, wherein steps 1 is carried out with the first enzyme *in vitro*, further comprising the steps after step 4 of:

contacting the candidate inhibitors selected in step 4 with a biological medium comprising whole cells having the first enzyme as an intracellular enzyme; and

selecting those candidate inhibitors which reduce the growth of the cells.

13. The method according to any previous claim, further comprising the step of:

using a selected inhibitor in a pharmaceutical preparation or a biocide.

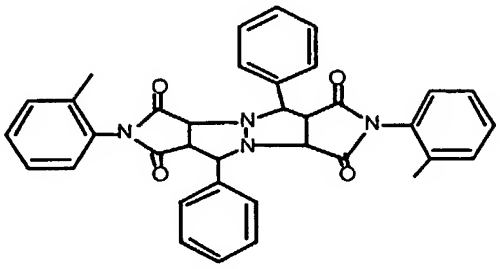
14. An inhibitor obtainable by the method of any of the claims 1 to 12.

15. An inhibitor for inhibiting in cells a cell enzyme converting a sugar phosphate into a sugar or a sugar alcohol phosphate into a sugar alcohol, the sugar or sugar alcohol being accumulated in large quantities by the cells for instance, but not exclusively, under conditions deviating from the optimal growth condition of the cells or as a reaction to stress conditions, the inhibition being either directly of the first enzyme or indirectly.

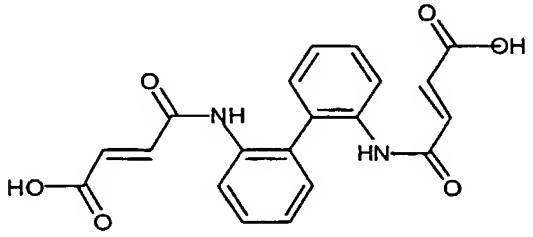
16. An inhibitor according to claims 14 or 15, wherein the inhibitor is an intracellular inhibitor.

17. An inhibitor according to any of claims 14 to 16, wherein the inhibitor specifically inhibits TPP enzymatic activity.

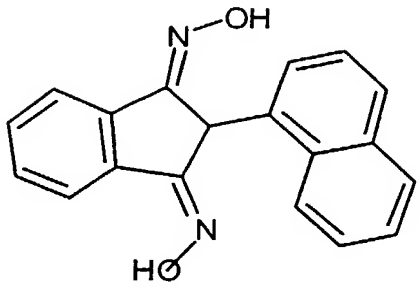
18. An inhibitor according to any of claims 14 to 17, whereby the inhibitor is a substance with the structure formula:



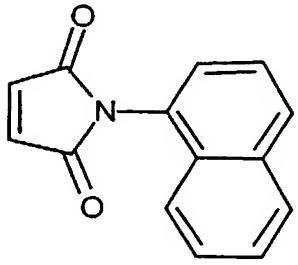
19. An inhibitor according to any of claims 14 to 17, whereby the inhibitor is a substance with the structure formula:



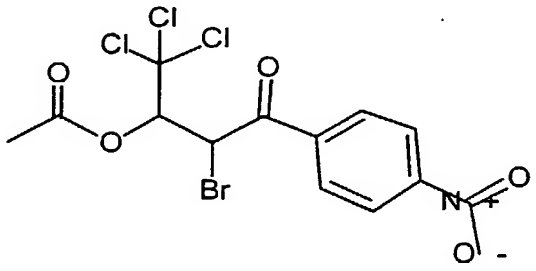
20. An inhibitor according to any of claims 14 to 17, whereby the inhibitor is a substance with the structure formula:



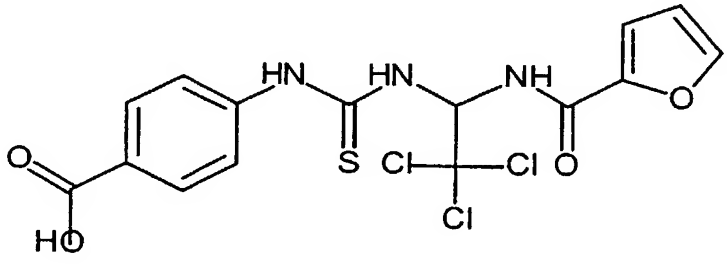
21. An inhibitor according to any of claims 14 to 17, whereby the inhibitor is a substance with the structure formula:



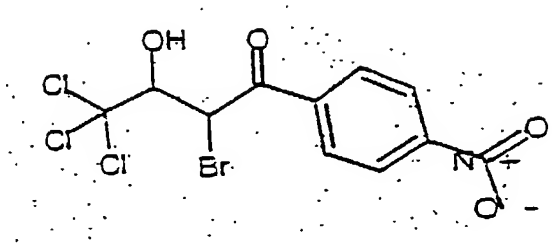
22. An inhibitor according to any of claims 14 to 17, whereby the inhibitor is a substance with the structure formula:



23. An inhibitor according to any of claims 14 to 17, whereby the inhibitor is a substance with the structure formula:



24. An inhibitor according to any of claims 14 to 17, whereby the inhibitor is a substance with the structure formula:



25. An inhibitor according to claims 14 to 17, whereby the inhibitor is any of the following substances identified in the DIVERSet™ library or derivatives or combinations thereof:

Compound Number	Position	Compound Number	Position
100764	PL 2 – D 4	155043	PL 41 – G 8
112710	PL 11 – H 4	155137	PL 41 – G 10
114854	PL 13 – A 10	153298	PL 42 – G 4
115308	PL 13 – D 7	156323	PL 44 – A 11
115800	PL 14 – A 2	158594	PL 44 – B 6
115806	PL 14 – A 5	159194	PL 46 – A 7
117003	PL 14 – F 6	159195	PL 46 – B 7
117033	PL 14 – G 6	159189	PL 46 – C 6
121911	PL 16 – H 2	156457	PL 46 – D 5
136794	PL 21 – H 5	156643	PL 46 – G 4
143067	PL 23 – A 9	159193	PL 46 – G 6
150050	PL 23 – H 2	157408	PL 46 – H 7
102330	PL 25 – A 3	140894	PL 46 – H 8
102341	PL 25 – A 7	155270	PL 46 – H 10
105299	PL 27 – E 9	156945	PL 47 – B 4
105463	PL 27 – G 10	106160	PL 47 – B 7
111087	PL 30 – A 2	122621	PL 47 – C 11
111189	PL 30 – A 8	155044	PL 47 – F 4
115781	PL 31 – A 8	159239	PL 47 – G 5
116321	PL 31 – B 7	161339	PL 47 – G 8
128067	PL 33 – G 8	105553	PL 47 – H 7

135235	PL 34 – G 10	108489	PL 47 – H 10
142159	PL 35 – A 5	104499	PL 48 – G 5
144152	PL 35 – B 5	169111	PL 49 – H 8
141531	PL 35 – C 3	182557	PL 51 – F 6
143530	PL 35 – C 5	113596	PL52-D3
141882	PL 35 – C 10	133207	PL 52 – E 10
143736	PL 35 – D 5	216645	PL 54 – H 4
136265	PL 35 – D 6	218940	PL 56 – A 2
142389	PL 35 – D 7	217973	PL 57 – H 4
136286	PL 35 – G 3	147933	PL 60 – A 4
141951	PL 35 – H 9	152078	PL 60 – E 9
143463	PL 36 – D 6	215222	PL 61 – C 10
143092	PL 36 – F 6	217342	PL 61 – F 10
143462	PL 36 – H 6	133445	PL 62 – E 7
104966	PL 38 – B 5	117172	PL 62 – F 5
118196	PL 38 – G 3	133805	PL 62 – G 7
107036	PL 38 – G 8	113610	PL 63 – A 8
150049	PL 41 – D 6	113222	PL 63 – B 3
153960	PL 41 – D 8	113233	PL 63 – C 3
145704	PL 41 – E 7	175327	PL 63 – F 6
146000	PL 41 – F 7	151234	PL 63 – H 3
151547	PL 41 – G 3		
146002	PL 41 – G 7		

26. An inhibitor according to any of claims 14 to 25, whereby the inhibitor is a biologically active salt, an ester derivative or a homolog of any of the compounds of claims 14 to 25 with the same functional activity.

27. An inhibitor according to any of claims 14 to 26 further comprising a stress raising factor for the target cell.

28. An inhibitor according to any of claims 14 to 27, wherein the inhibiting effect is greater that that of NEM and/or of DTNB.

29. A pharmaceutical preparation comprising one or more of the inhibitors of any of claims 14 to 28.

30. The pharmaceutical preparation according to claim 29, further comprising a drug active against a parasite having a biosynthetic pathway converting a sugar phosphate into a sugar or a sugar alcohol phosphate into a sugar alcohol.

31. The pharmaceutical preparation of claim 30, wherein the drug is an azole.

32. The pharmaceutical preparation of claim 30, wherein the drug is one of Amphotericin B, Flucytosine, Ketoconazole, Miconazole, Fluconazole, and Itraconazole.

33. The pharmaceutical preparation according to any of claims 29 to 32, further comprising a stress raising factor for the target pathogen.

34. A biocide acting on fungi, insects, nematodes, bacteria or other organisms accumulating large quantities of a sugar alcohol or a sugar in response to stress comprising the inhibitor of any of claims 14 to 28.

35. The biocide of claim 34, further comprising an agent against a parasite having a biosynthetic pathway converting a sugar phosphate into a sugar or a sugar alcohol phosphate into a sugar alcohol.

36. The biocide of claim 35 wherein the agent is an azole.

37. A method of increasing the sugar phosphate or sugar alcohol phosphate content in a target cell, comprising the step of:

reducing the activity of a first cell enzyme converting a sugar phosphate into a sugar or a sugar alcohol phosphate into a sugar alcohol in the target cells, by using an inhibitor.

38. A method of increasing the sugar phosphate or sugar alcohol phosphate content in a mammalian parasite, comprising the step of:

reducing the activity of a first cell enzyme converting a sugar phosphate into a sugar or a sugar alcohol phosphate into a sugar alcohol in cells of the parasite.

39. A method of increasing the sugar phosphate or sugar alcohol phosphate content in a mammalian parasite, comprising the step of:

reducing or inhibiting the activity of a first cell enzyme converting a sugar phosphate into a sugar or a sugar alcohol phosphate into a sugar alcohol in cells of the parasite, by a single or double knockout deletion mutation of the parasite for that enzyme.

40. A method of reducing or impairing the pathogenecity of a mammalian parasite by promoting hyperaccumulation of a sugar phosphate or a sugar alcohol phosphate.

Tps1
UDP-glucose + Glucose-6-P
Tps2
trehalose-6-P
trehalose
Tps1: trehalose-6-P synthase
Tps2: trehalose-6-P phosphatase
Tps3/ Tsl1
Tps1
Tps2
Tps1
UDP-glucose + Glucose-6-P
Tps2
(trehalose-6-P)
(trehalose)
Tps1Δ
Tps1
UDP-glucose + Glucose-6-P
Tps2
trehalose-6-P
Tps2Δ
trehalose

Fig. 1

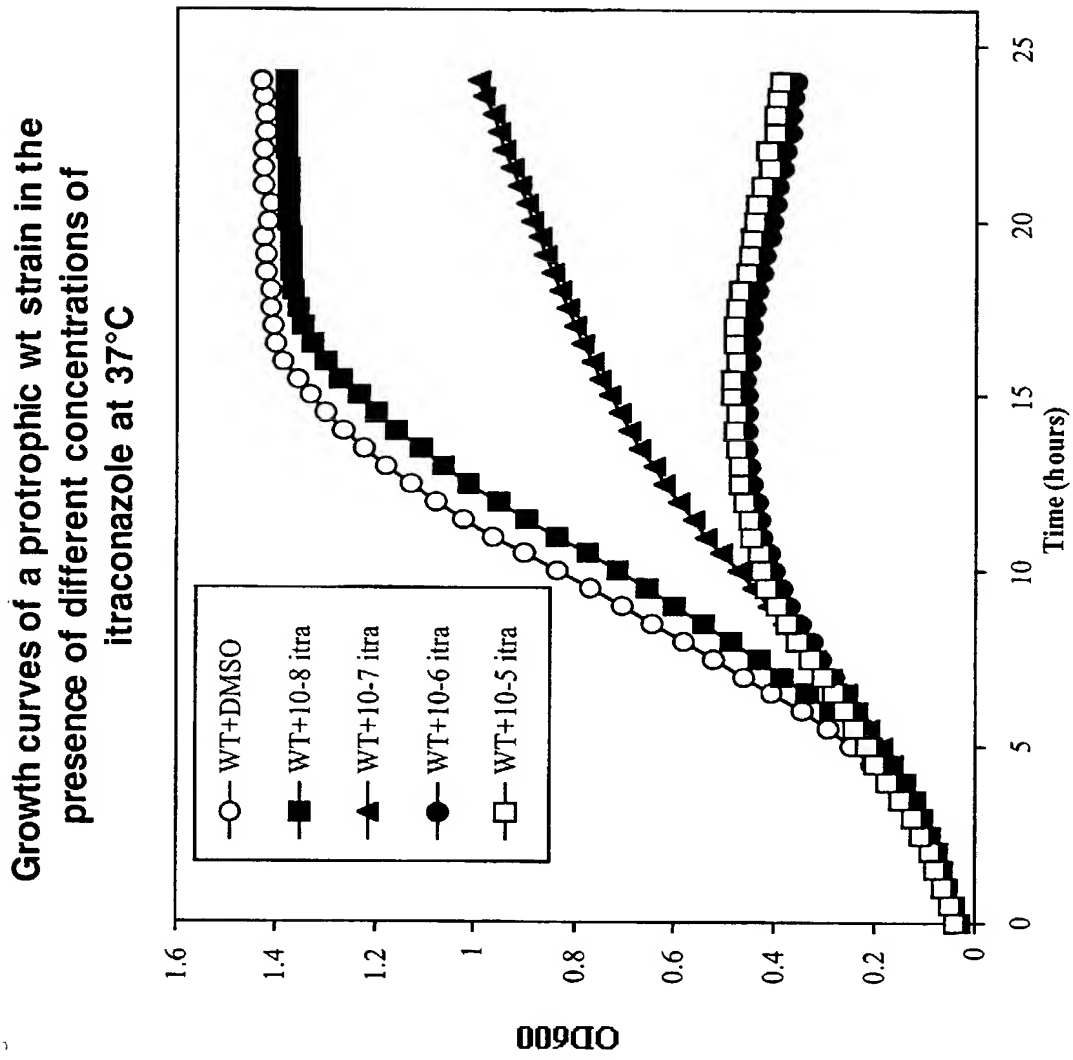

Growth curves of a protrophic wt strain in the presence of different concentrations of itraconazole at 37°C
WT+DMSO
WT+10-8 itra
WT+10-7 itra
WT+10-6 itra
WT+10-5 itra
OD600
Time (hours)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
0
5
10
15
20
25

Fig. 2A

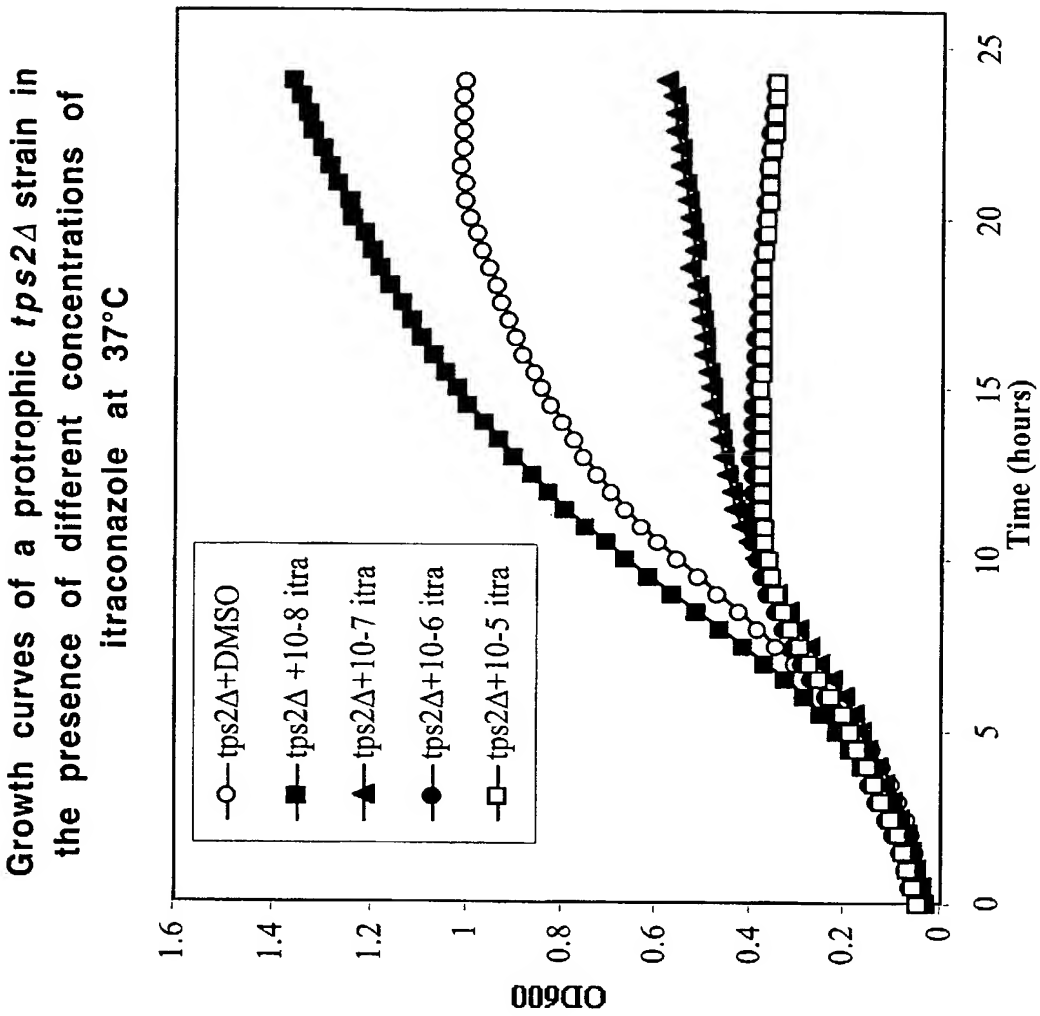

Growth curves of a protrophic tps2Δ strain in the presence of different concentrations of itraconazole at 37°C
tps2Δ+DMSO
tps2Δ +10-8 itra
tps2Δ+10-7 itra
tps2Δ+10-6 itra
tps2Δ+10-5 itra
OD600
Time (hours)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
5
10
15
20
25

Fig. 2B

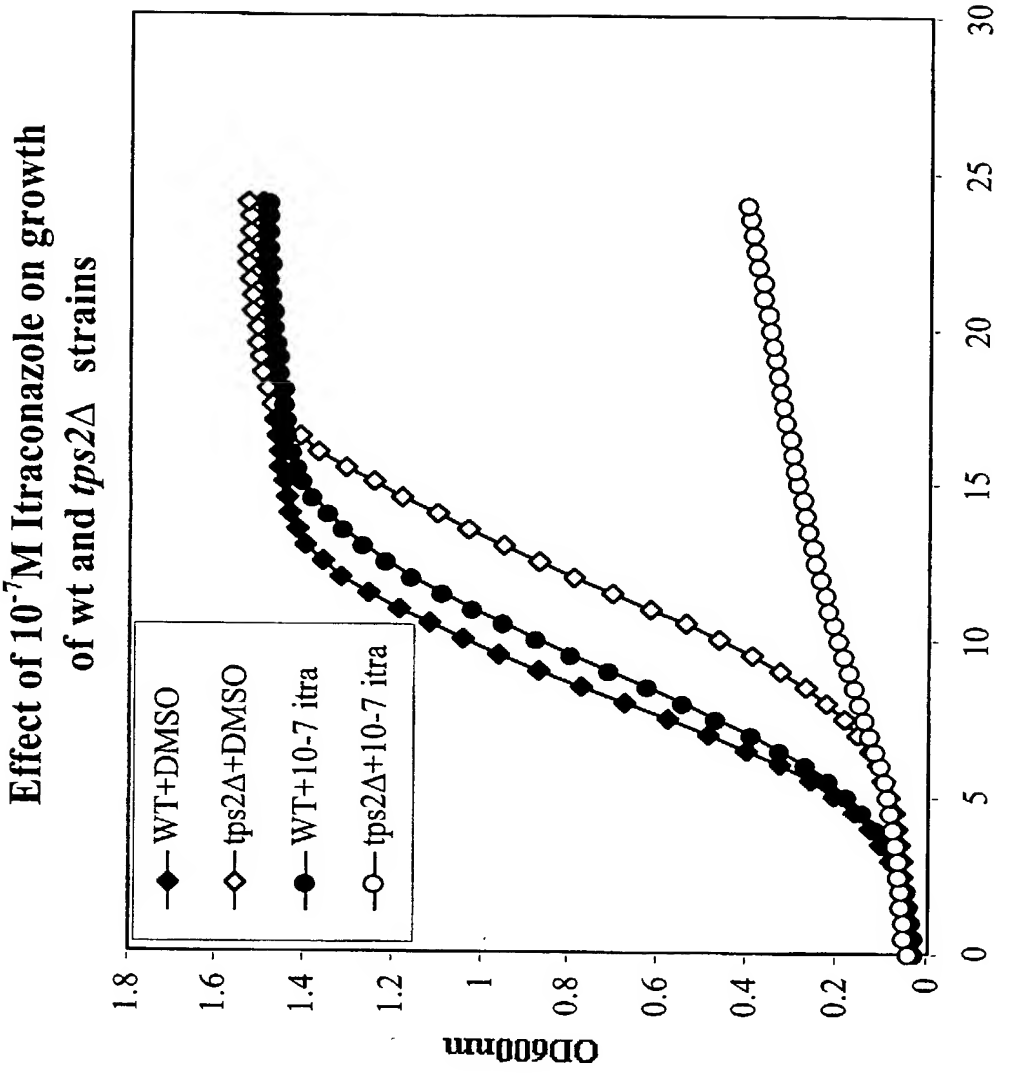

Effect of 10^{-7}M Itraconazole on growth of wt and *tps2Δ* strains
WT+DMSO
tps2Δ+DMSO
WT+10-7 itra
tps2Δ+10-7 itra
OD600nm
Time (hours)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
0
5
10
15
20
25
30

Fig. 3

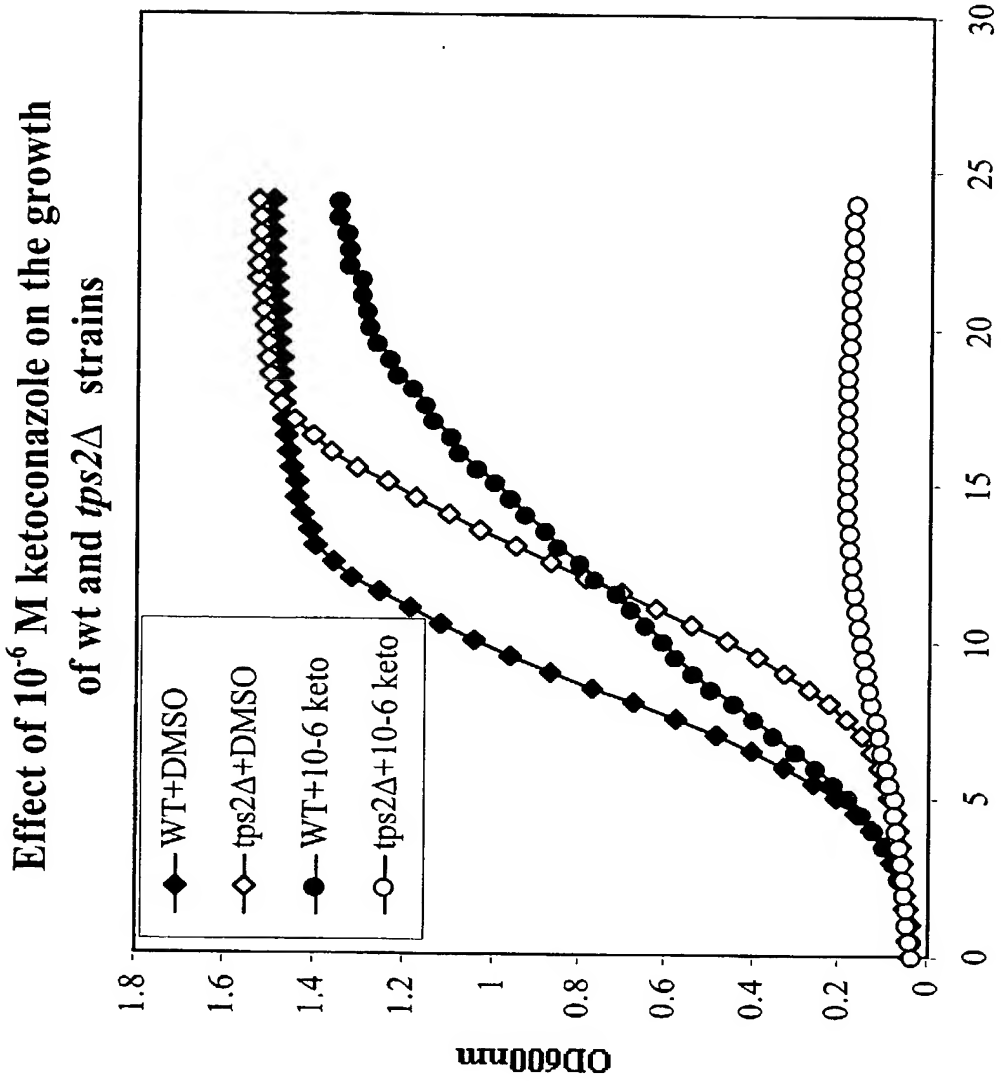

Effect of 10^{-6} M ketoconazole on the growth of wt and *tps2Δ* strains
WT+DMSO
tps2Δ+DMSO
WT+10-6 keto
tps2Δ+10-6 keto
OD600nm
Time (hours)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
0
5
10
15
20
25
30

Fig. 4

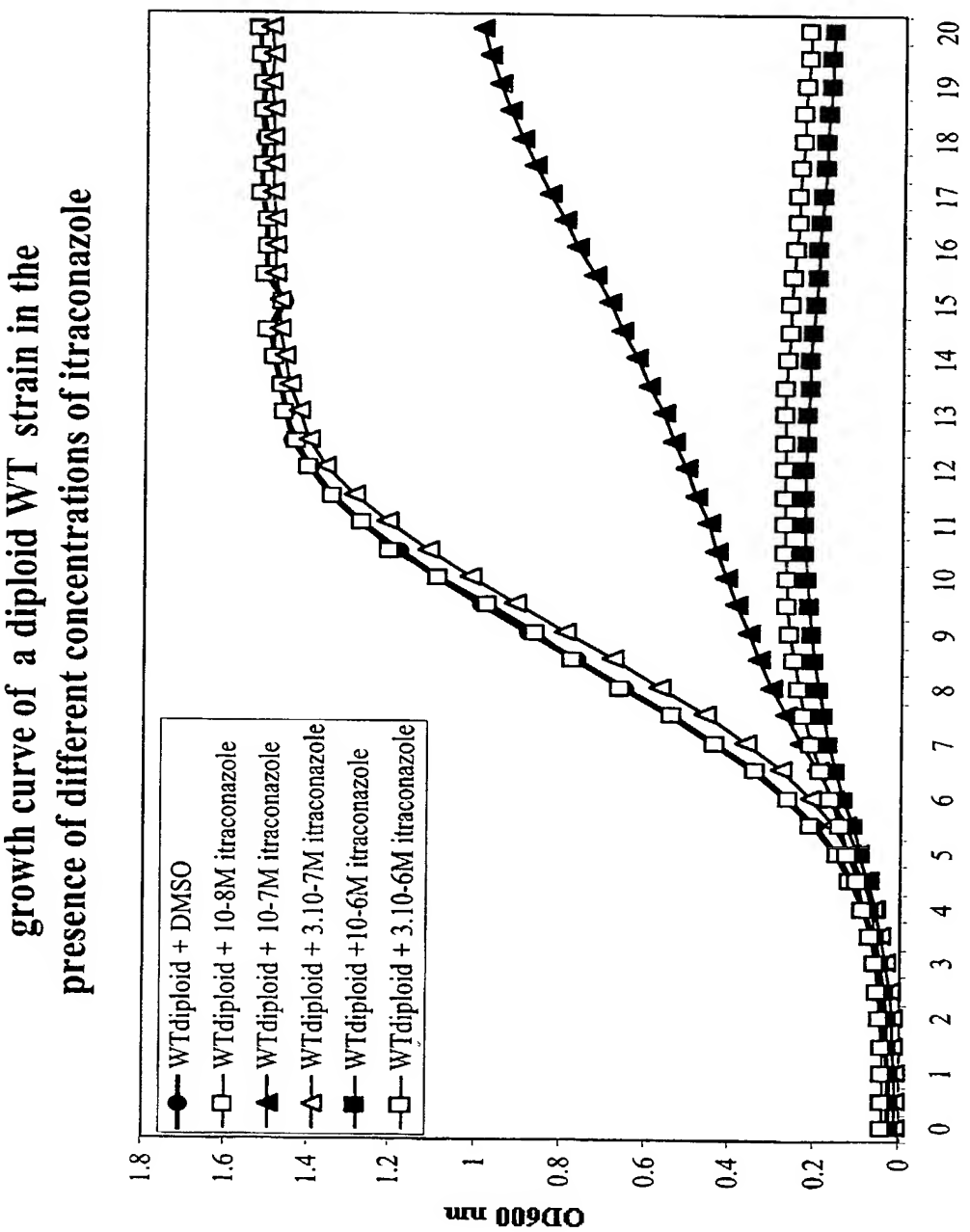

growth curve of a diploid WT strain in the presence of different concentrations of itraconazole
WTdiploid + DMSO
WTdiploid + 10-8M itraconazole
WTdiploid + 10-7M itraconazole
WTdiploid + 3.10-7M itraconazole
WTdiploid +10-6M itraconazole
WTdiploid + 3.10-6M itraconazole
OD600 nm
Time (hours)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Fig. 5A

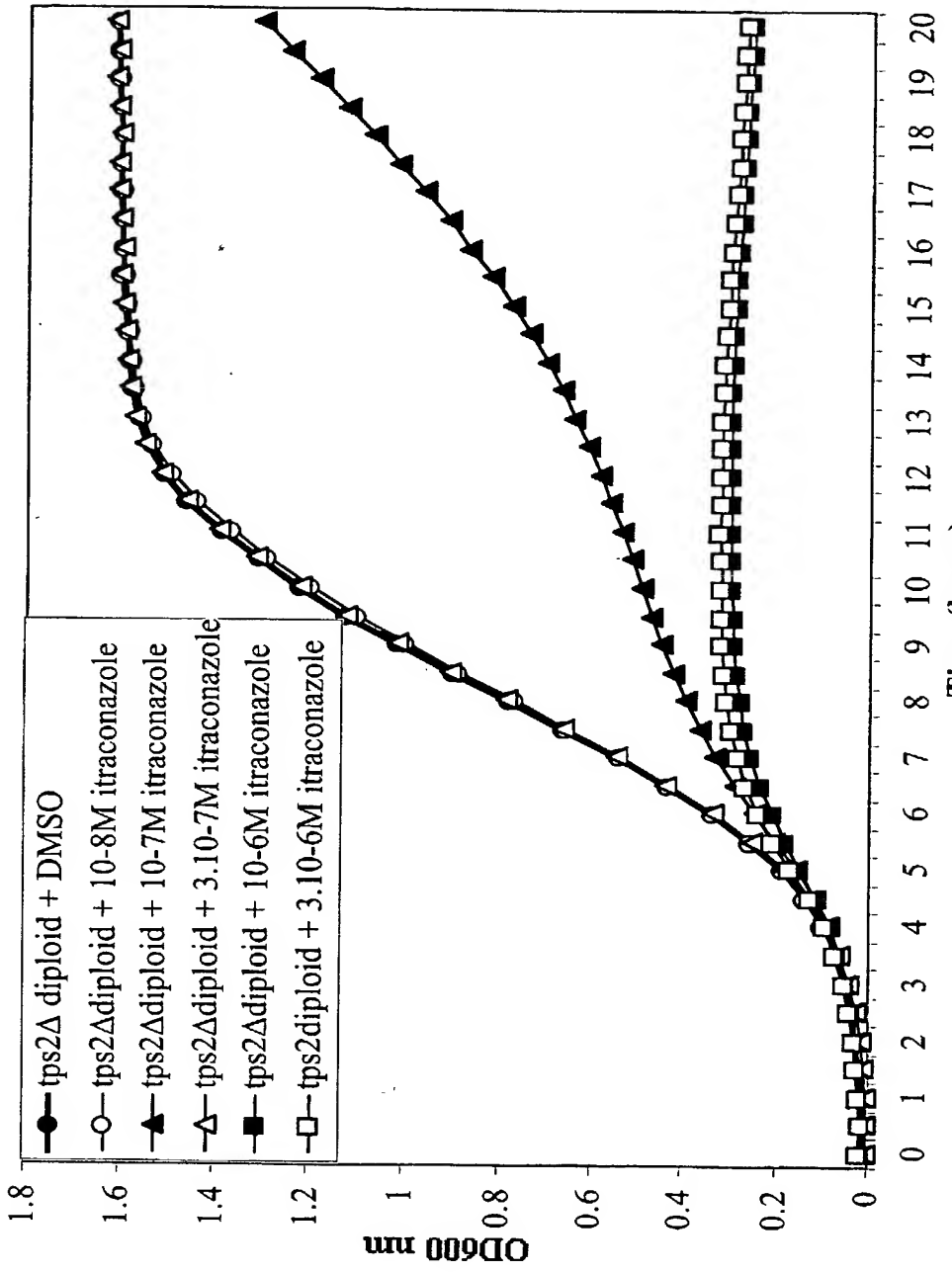

growth curve of a diploid heterozygous tps2Δ strain
in the presence of different concentrations of itraconazole
tps2Δ diploid + DMSO
tps2Δdiploid + 10-8M itraconazole
tps2Δdiploid + 10-7M itraconazole
tps2Δdiploid + 3.10-7M itraconazole
tps2Δdiploid + 10-6M itraconazole
tps2diploid + 3.10-6M itraconazole
OD600 nm
Time (hours)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Fig. 5B

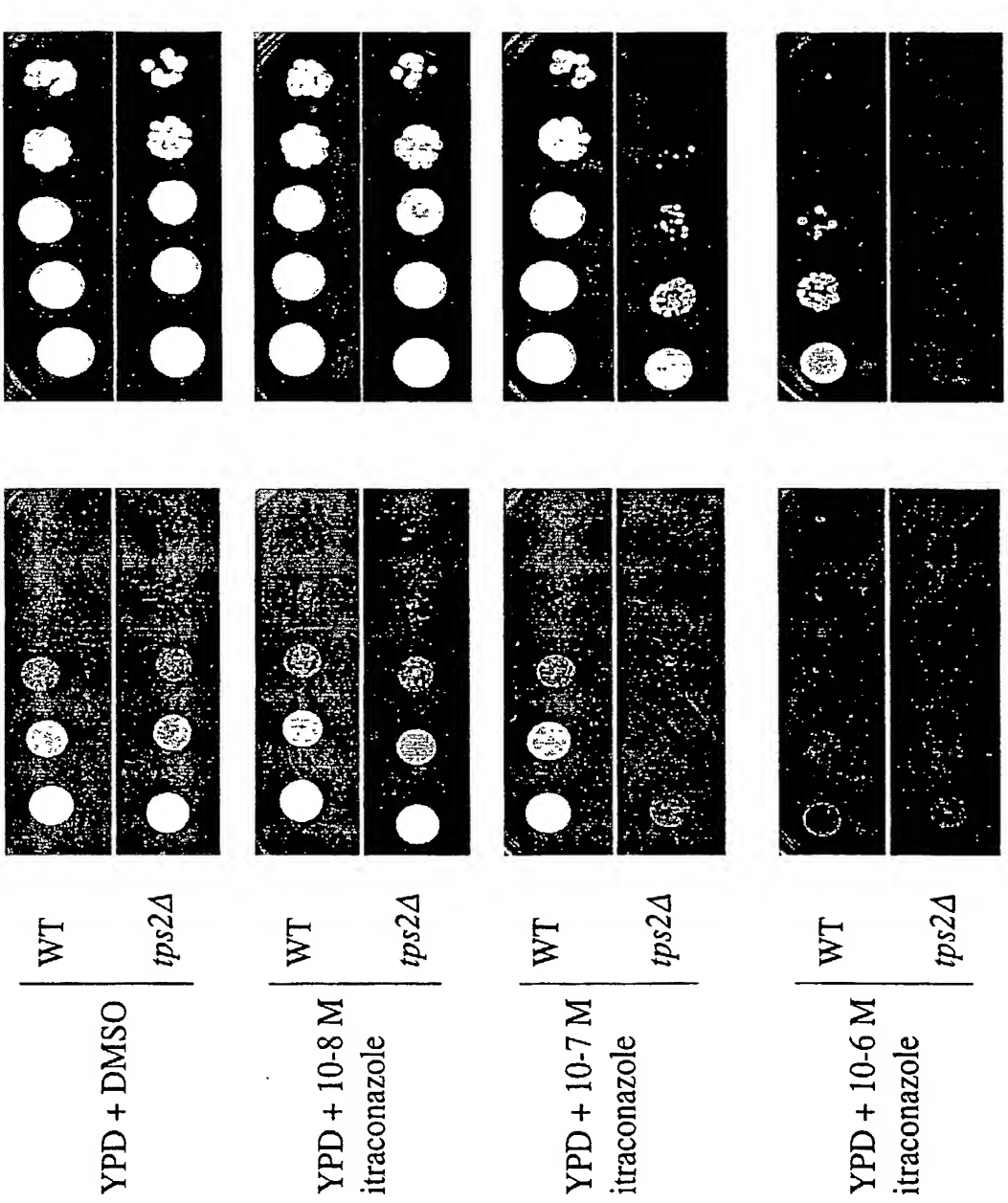

After 1 day incubation
After 2 days incubation
YPD + DMSO
YPD + 10-8 M itraconazole
YPD + 10-7 M itraconazole
YPD + 10-6 M itraconazole
WT
tps2Δ

Fig. 6

There was no growth of the strains on YPD at 41 °C

Fig. 7

```
S.cerevisiaeTPS2  ----------------MTTTAQDNSPKKRQRIINCVTQLPYKIQLGESNDD----WKIS 39
C.albicans        MAPQQVNAFSANGSIPSPKEFEGKGKLKLSGRILNVMTSLPLQIISDYDNKTGQYYWDVE 60
                                .  |  ...  *   **:* :*.** :*   . .*.      *.:.
S.cerevisiaeTPS2  ATTGNSALYSSLEYLQFDSTEYEQHVVGWTGEITRTERNLFTREAKEKPQDLDDDPLYLT 99
C.albicans        TVRGNSALYSSQHFLAEN-KEWETHLIAWTGELINKKKDTSSLTADT----LQDDPLYLD 115
                  :. ******** .:*  : .*:* *::.****: ..::: :  *.      *:******
                     |
S.cerevisiaeTPS2  KEQINGLTTTLQDHMKSDKEAKTDTTQTAPVTNNVHPVWLLRKNQSRWRNYAEKVIWPTF 159
C.albicans        EEDKLKIEKKLCDASG----------TPN---IHPVWLLRRDQGRWRKYAENVLWPVF 160
                  :*:  : ..* *              :*    :********::*.***:***:*:**.*
                     |
S.cerevisiaeTPS2  HYILNPSNEGEQEKNWWYDYVKFNEAYAQKIGEVYRKGDIIWIHDYYLLLLPQLLRMKFN 219
C.albicans        HYIQGQPSDGKAETDAWHDYVKFNEAYLNKIKSVYKPGDIIWIHDYYLLLLPQLLRMEFP 220
                  *** . ..:*: *.: *:********** :** .**: ************************:*
                     |
S.cerevisiaeTPS2  DESIIIGYFHHAPWPSNEYFRCLPRRKQILDGLVGANRICFQNESFSRHFVSSCKRLLDA 279
C.albicans        N--AYIGFFLHVPFPSSEYFRCLSKRSQLLDGMLGADKIGFQSDSFQRHFISCCARVLGC 278
                  :   **:* *.*:**.****.**.:*.*:***::**:* **.:**.***:*.* *:*..
                     |
S.cerevisiaeTPS2  TAKKSKNSSDSDQYQVSVYGGDVLVDSLPIGVNTTQILKDAFT--KDIDSKVLSIKQAYQ 337
C.albicans        EVNR--D-------SVSAYGTTISVETLPIGIDTEKIEHDAFSSELGVEEKVQALKQVYK 329
                  .:: :       .**.**  : *:;****::* :* :***:    .::.** ::**.*:
                     |
S.cerevisiaeTPS2  NKKIIIGRDRLDSVRGVVQKLRAFETFLAMYPEWRDQVVLIQVSSPTANRNSPQTIRLEQ 397
C.albicans        GKKLIVGRDRLDKVRGVIQKLEGFEIFLDMYPEWRETVVLIQVSSPGYS-HSAN--VET 385
                  .**:*:*******.****:***..** ** *******: **********   . :*.:  :*
                     |
S.cerevisiaeTPS2  QVNELVNSINSEYGNLNFSPVQHYYMRIPKDVYLSLLRVADLCLITSVRDGMNTTALEYV 457
C.albicans        RVTEIISRINSKYGNLNHTPVLHYQMRVAKEEYLALLRVADLALITSVRDGMNTTSLEFV 445
                  :*.*::. ***:*****.:** ** **:.*: **:********.**************:**:*
                     |
S.cerevisiaeTPS2  TVKSHMSNFLCYGNPLILSEFSGSSNVLKDAIVVNPWDSVAVAKSINMALKLDKEEKSNL 517
C.albicans        ICQKYN-N-----SPLILSEFTGTATVLKDAIMVNPWDSVGVAKTINDALMLSTKEKVSL 499
                    :.:  *     .*******:*::.*******:*******.***:** ** *..:** .*
```

Fig. 8A

```
S.cerevisiaeTPS2  ESKLWKEVPTIQDWTNKFLSSLKEKASSDDDVERKMTPALNRPVLLENYKQAKRRLFLFD 577
C.albicans        ESKLYEKVLSNTVQNWTSTFICDILSHSIVTHSNSYTPALNRPLLLNNYKESQRRLFLFD 559
                  ****:::* :    .  .     .  : *     ...  ********:**:***:::******

S.cerevisiaeTPS2  YDGTLTPIVKDPAAAIPSARLYTILQKLCADPHNQIWIISGRDQKFLNKWLGGKLPQLGL 637
C.albicans        YDGTLTPIVQDPAAAIPSDKLNRILDVLSSDPKNQIWIISGRDQAFLEKWMGNKN--VGL 617
                  **********:*********** :*  **: *.:**:************ **:**:*.*   :**

S.cerevisiaeTPS2  SAEHGCFMKDVSCQDWVNLTEKVDMSWQVRVNEVMEEFTRTPGSFIERKKVALTWHYRR 697
C.albicans        SAEHGCFMKDIGSKEWVNLAASFDMSWQEKVDDIFKYYTEKTPGSNIERKKVALTWHYRR 677
                  **********:..::****: ..***** :*::::: :* :**** ****************

S.cerevisiaeTPS2  TVPELGEFHAKELKEKLLS-FTDDFDLEVMDGKANIEVRPRFVNKGEIVKRLVWHQHGKP 756
C.albicans        ADPDLGNFQAEKCMKELNDTVAKEYDVEVMAGKANIEVRPKFVNKGEIVKRLVLHPHGAK 737
                  : *:**:*:*::  ::* . .::::*:*** ********** :************ * **

S.cerevisiaeTPS2  QDMLKG--ISEKLPKDEMPDFVLCLGDDFTDEDMFRQLNTIETCWKEKYPDQKNQWGNYG 814
C.albicans        QEKHPTGHCTKDIPIEELPDFMLCLGDDLTDEDMFNSLNEINKKWKGDN-RPTNKFGSYG 796
                  *;      ::.:* :*:***:*******:******..** *:.  **  .    .*::*.**

S.cerevisiaeTPS2  FYPVTVGSASKKTVAKAHLTDPQQVLETLGLLVGDVSLFQSAGTVDLDSRGHVKNSESSL 874
C.albicans        VYPVAVGPASKKTVAIAHLNEPRQVLETLGLLAGLVSIFESAGTVDLDDRVTLPIVCLPK 856
                  .***:**.*******  ***.:*:**********.* **:*:*********.*  :    .

S.cerevisiaeTPS2  KSKLASKAYVMKRSASYTGAKV 896
C.albicans        DQTMLYLRQYLYVKKLVKKKKL 878
                  ...:    :  .  .  *:
```

Fig. 8B

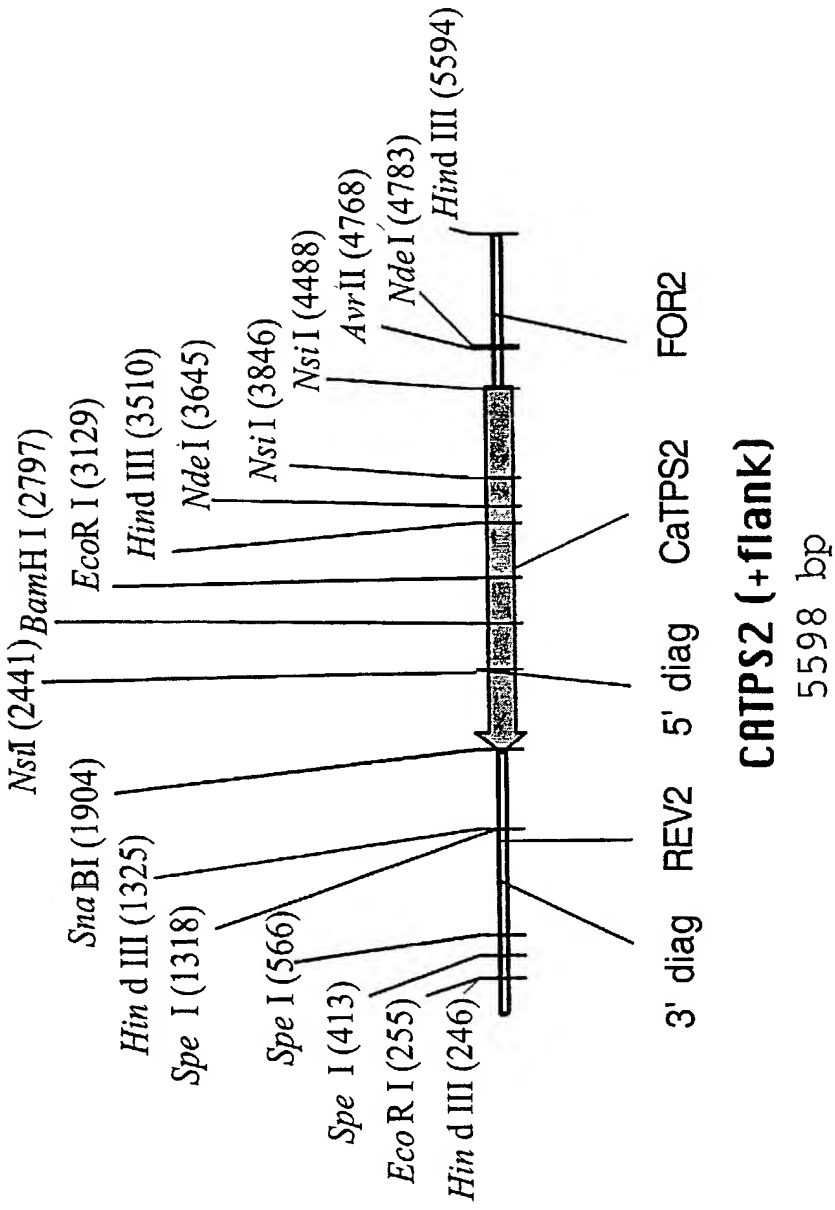

NsiI (2441)
BamH I (2797)
EcoR I (3129)
Hind III (3510)
Nde I (3645)
Nsi I (3846)
Nsi I (4488)
Avr II (4768)
Nde I (4783)
Hind III (5594)
Sna BI (1904)
Hin d III (1325)
Spe I (1318)
Spe I (566)
Spe I (413)
Eco R I (255)
Hin d III (246)
3' diag
REV2
5' diag
CaTPS2
FOR2
CATPS2 (+flank)
5598 bp

Fig. 9

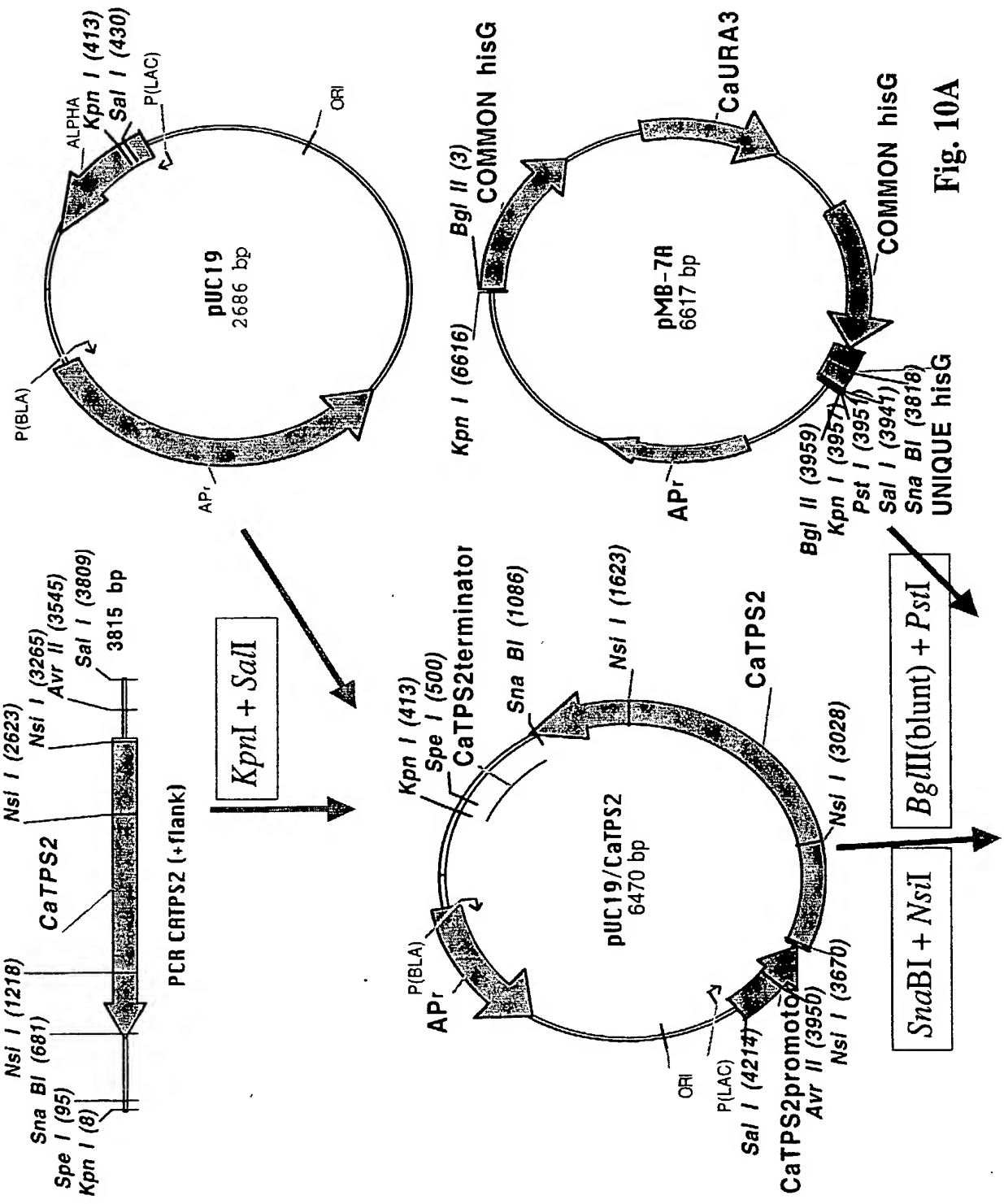

pUC19
2686 bp
P(BLA)
APr
ORI
ALPHA
Kpn I (413)
Sal I (430)
P(LAC)
PCR CATPS2 (+flank)
Nsi I (2623)
Nsi I (3265)
Avr II (3545)
Sal I (3809)
3815 bp
CaTPS2
Nsi I (1218)
Sna BI (681)
Spe I (95)
Kpn I (8)
KpnI + SalI
pMB-7R
6617 bp
Bgl II (3)
COMMON hisG
Kpn I (6616)
CaURA3
APr
COMMON hisG
Bgl II (3959)
Kpn I (3957)
Pst I (3950)
Sal I (3941)
Sna BI (3818)
UNIQUE hisG
pUC19/CaTPS2
6470 bp
Kpn I (413)
Spe I (500)
CaTPS2terminator
Sna BI (1086)
Nsi I (1623)
CaTPS2
Nsi I (3028)
APr
P(BLA)
ORI
P(LAC)
Sal I (4214)
CaTPS2promotor
Avr II (3950)
Nsi I (3670)
SnaBI + NsiI
BglII(blunt) + PstI

Fig. 10A

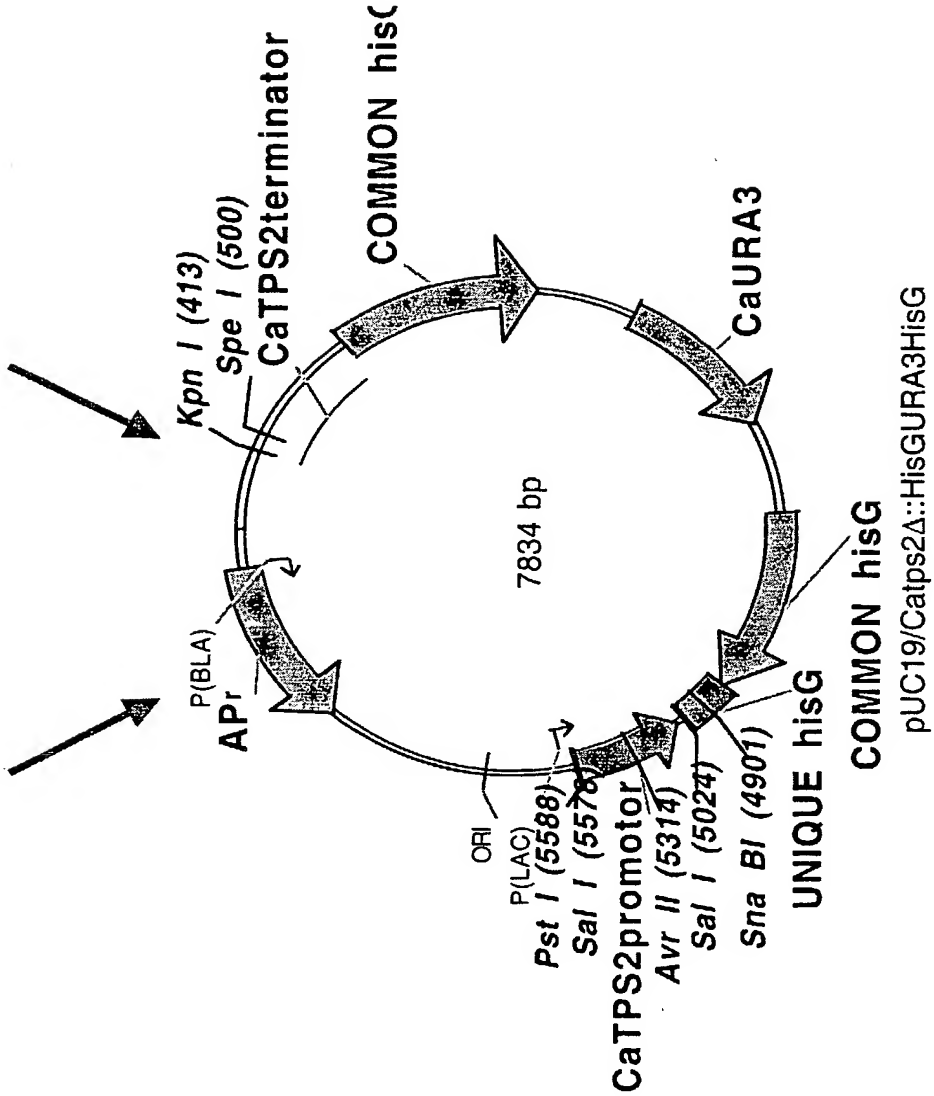

Kpn I (413)
Spe I (500)
CaTPS2terminator
COMMON hisG
CaURA3
7834 bp
P(BLA)
APr
ORI
P(LAC)
Pst I (5588)
Sal I (5578)
CaTPS2promotor
Avr II (5314)
Sal I (5024)
Sna BI (4901)
UNIQUE hisG
COMMON hisG
pUC19/Catps2Δ::HisGURA3HisG

Fig. 10B

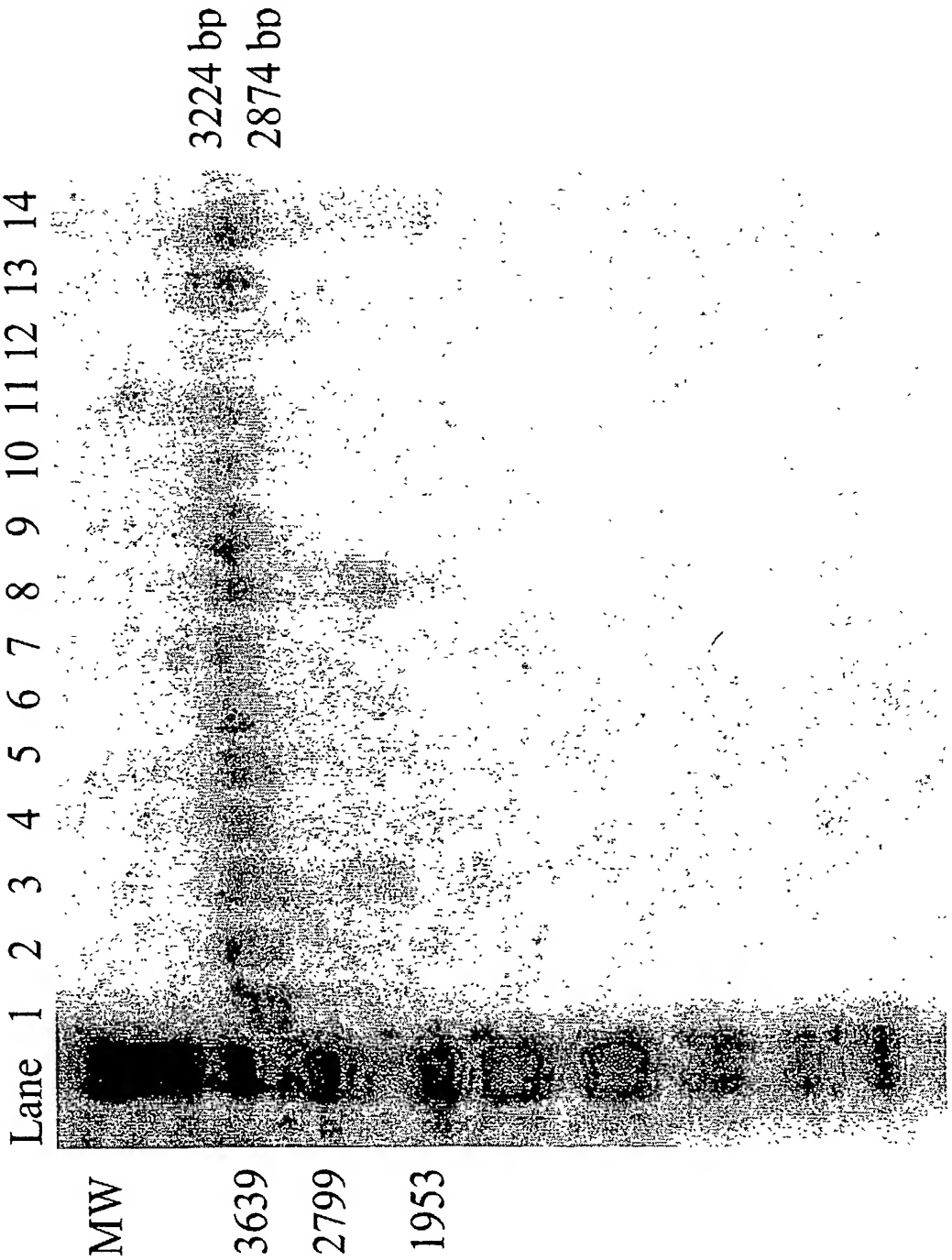


Fig. 11

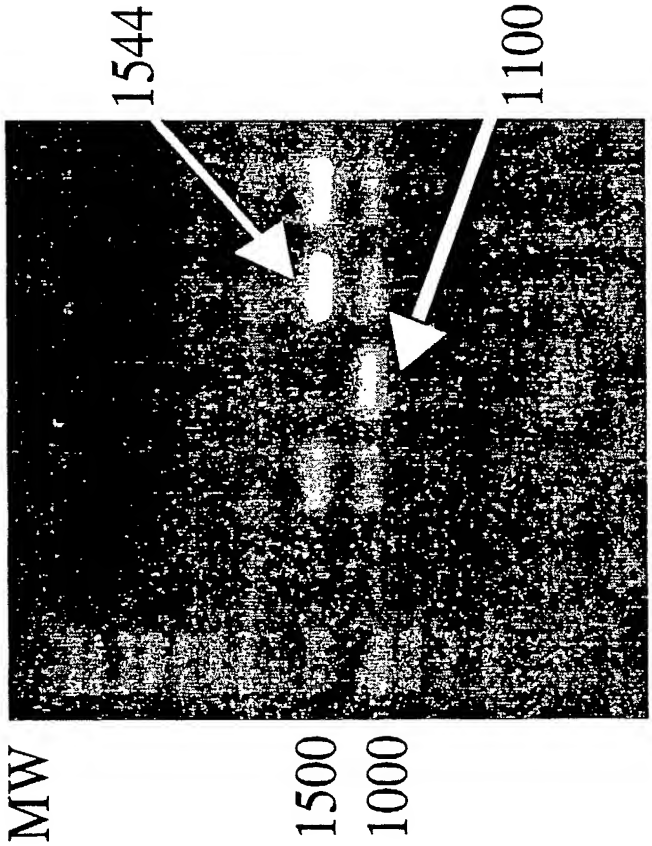


Fig. 12A

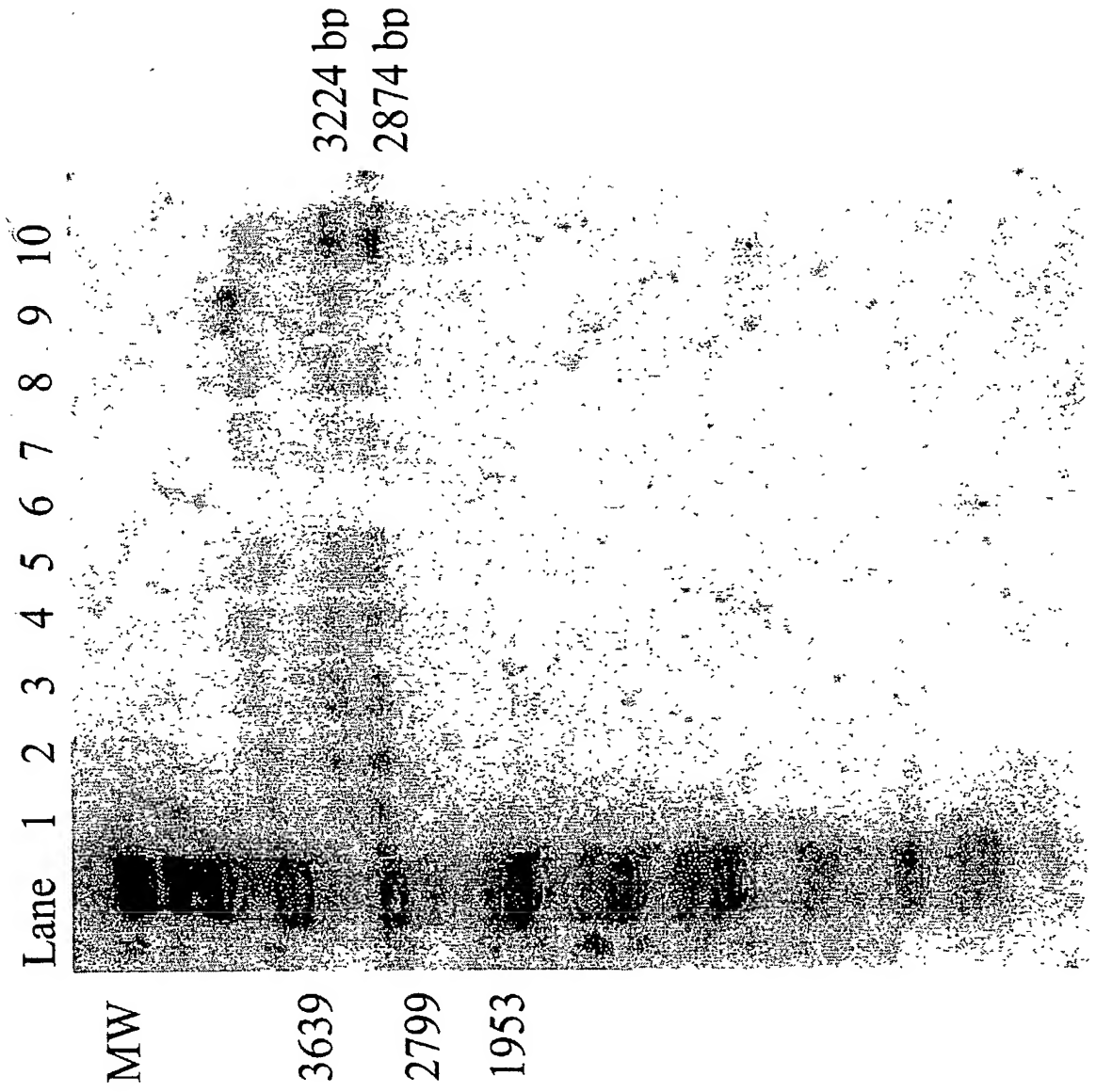


Fig. 12B

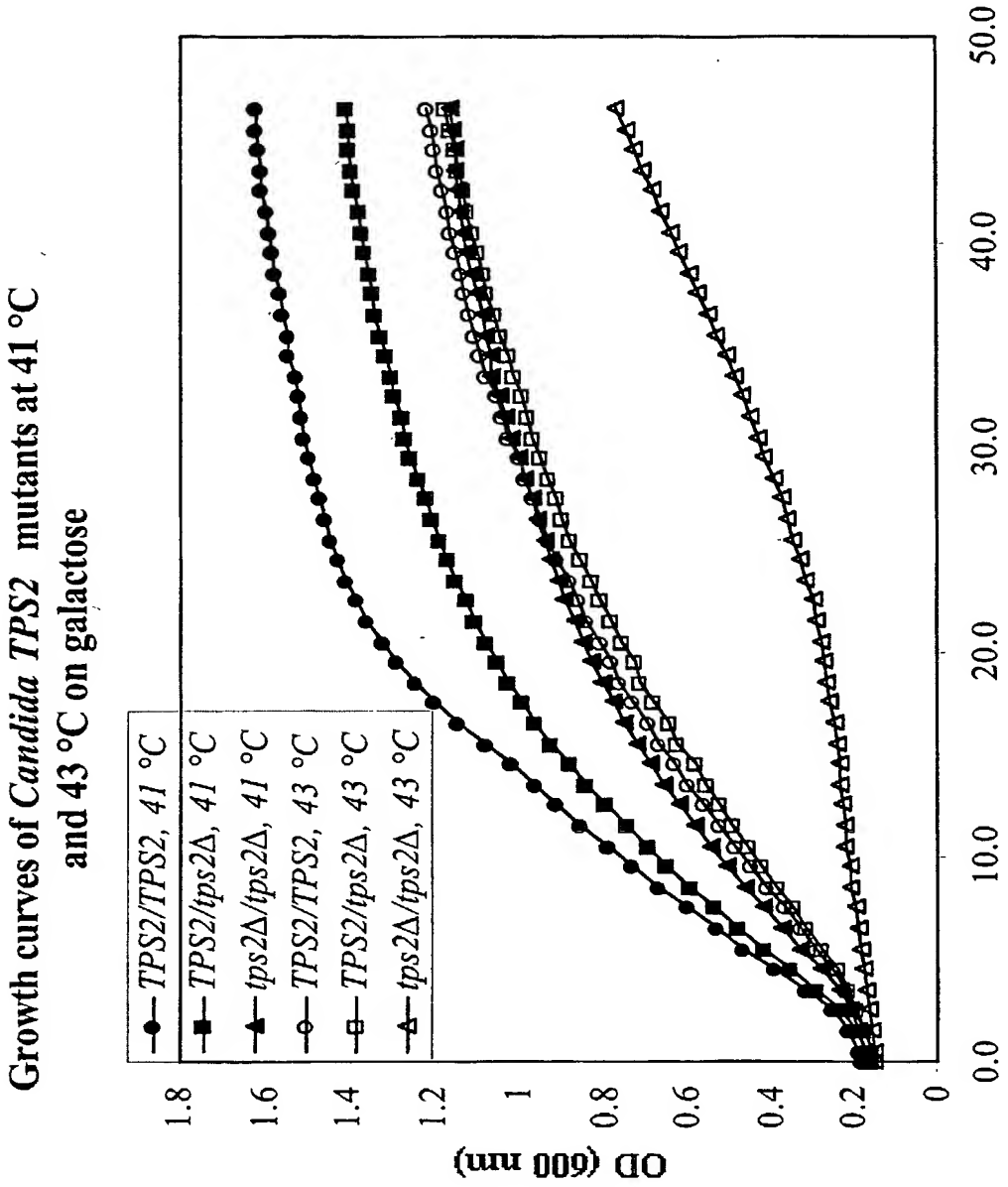

Growth curves of Candida TPS2 mutants at 41 °C and 43 °C on galactose
TPS2/TPS2, 41 °C
TPS2/tps2Δ, 41 °C
tps2Δ/tps2Δ, 41 °C
TPS2/TPS2, 43 °C
TPS2/tps2Δ, 43 °C
tps2Δ/tps2Δ, 43 °C
OD (600 nm)
Time (hours)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
0.0
10.0
20.0
30.0
40.0
50.0

Fig. 13A

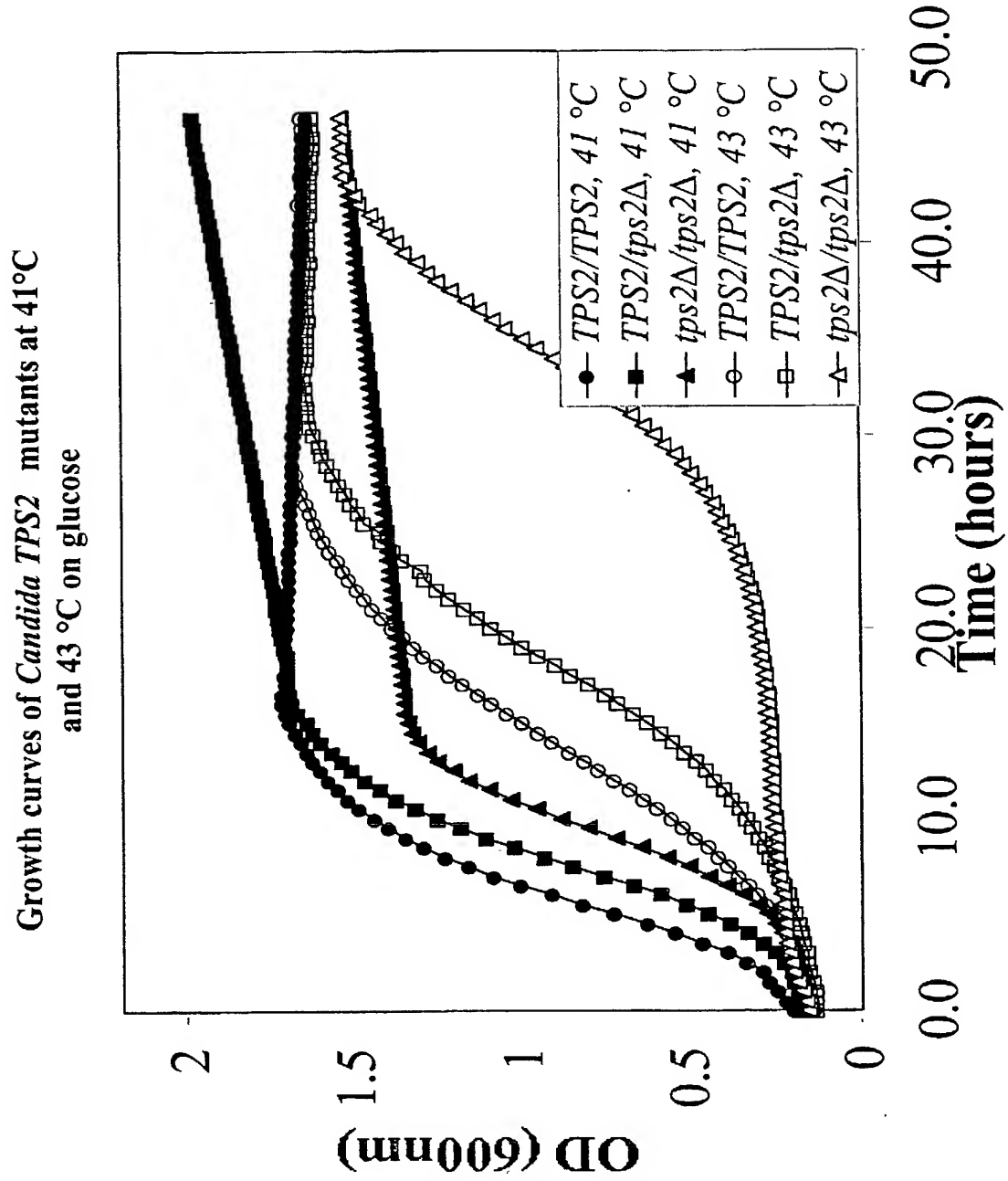

Growth curves of Candida TPS2 mutants at 41°C and 43 °C on glucose
OD (600nm)
Time (hours)
0
0.5
1
1.5
2
0.0
10.0
20.0
30.0
40.0
50.0
TPS2/TPS2, 41 °C
TPS2/tps2Δ, 41 °C
tps2Δ/tps2Δ, 41 °C
TPS2/TPS2, 43 °C
TPS2/tps2Δ, 43 °C
tps2Δ/tps2Δ, 43 °C

Fig. 13B

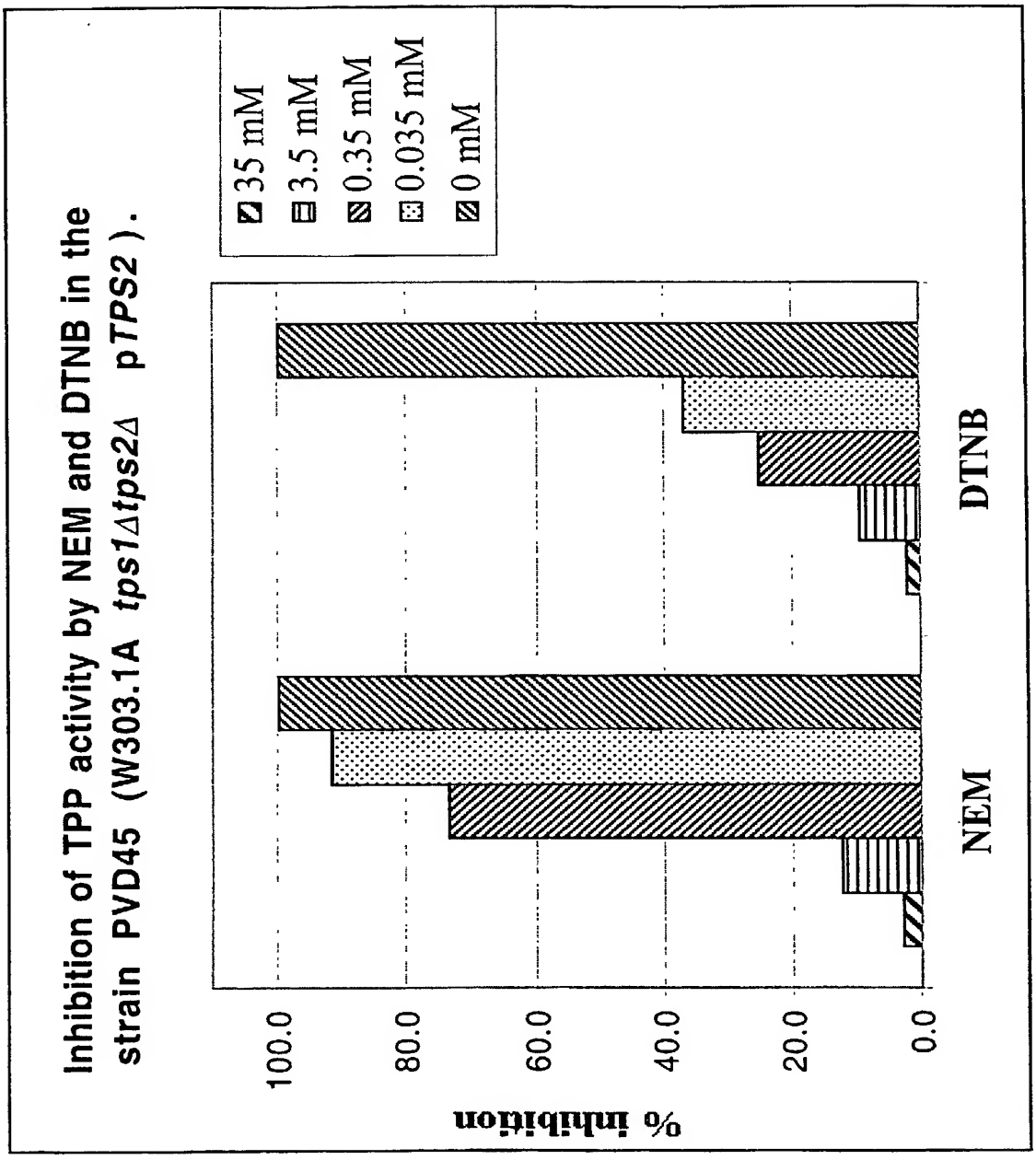

Inhibition of TPP activity by NEM and DTNB in the strain PVD45 (W303.1A tps1Δtps2Δ pTPS2).
35 mM
3.5 mM
0.35 mM
0.035 mM
0 mM
% inhibition
100.0
80.0
60.0
40.0
20.0
0.0
NEM
DTNB

Fig.14

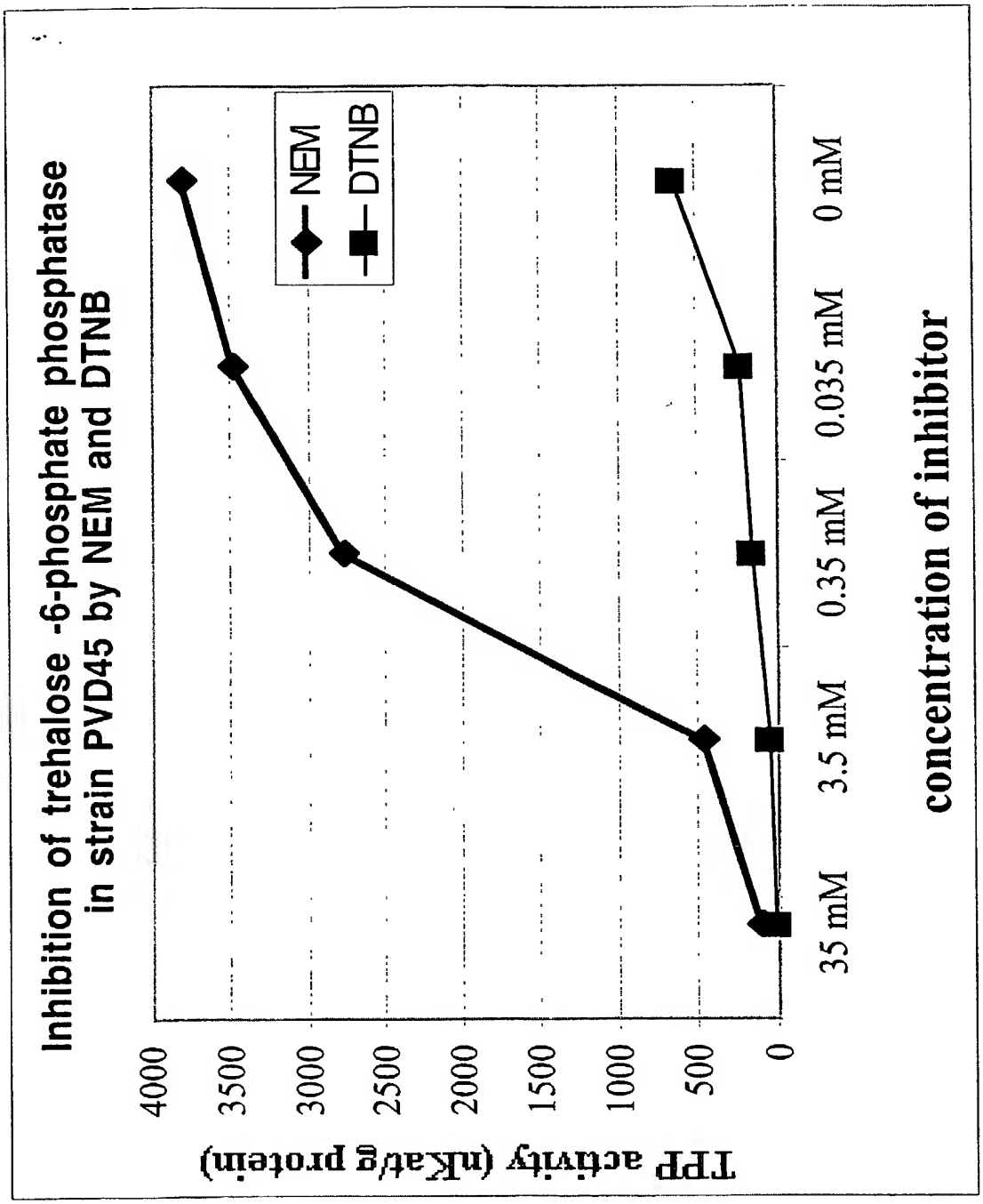

Inhibition of trehalose -6-phosphate phosphatase in strain PVD45 by NEM and DTNB
NEM
DTNB
TPP activity (nKat/g protein)
4000
3500
3000
2500
2000
1500
1000
500
0
35 mM
3.5 mM
0.35 mM
0.035 mM
0 mM
concentration of inhibitor

Fig. 15

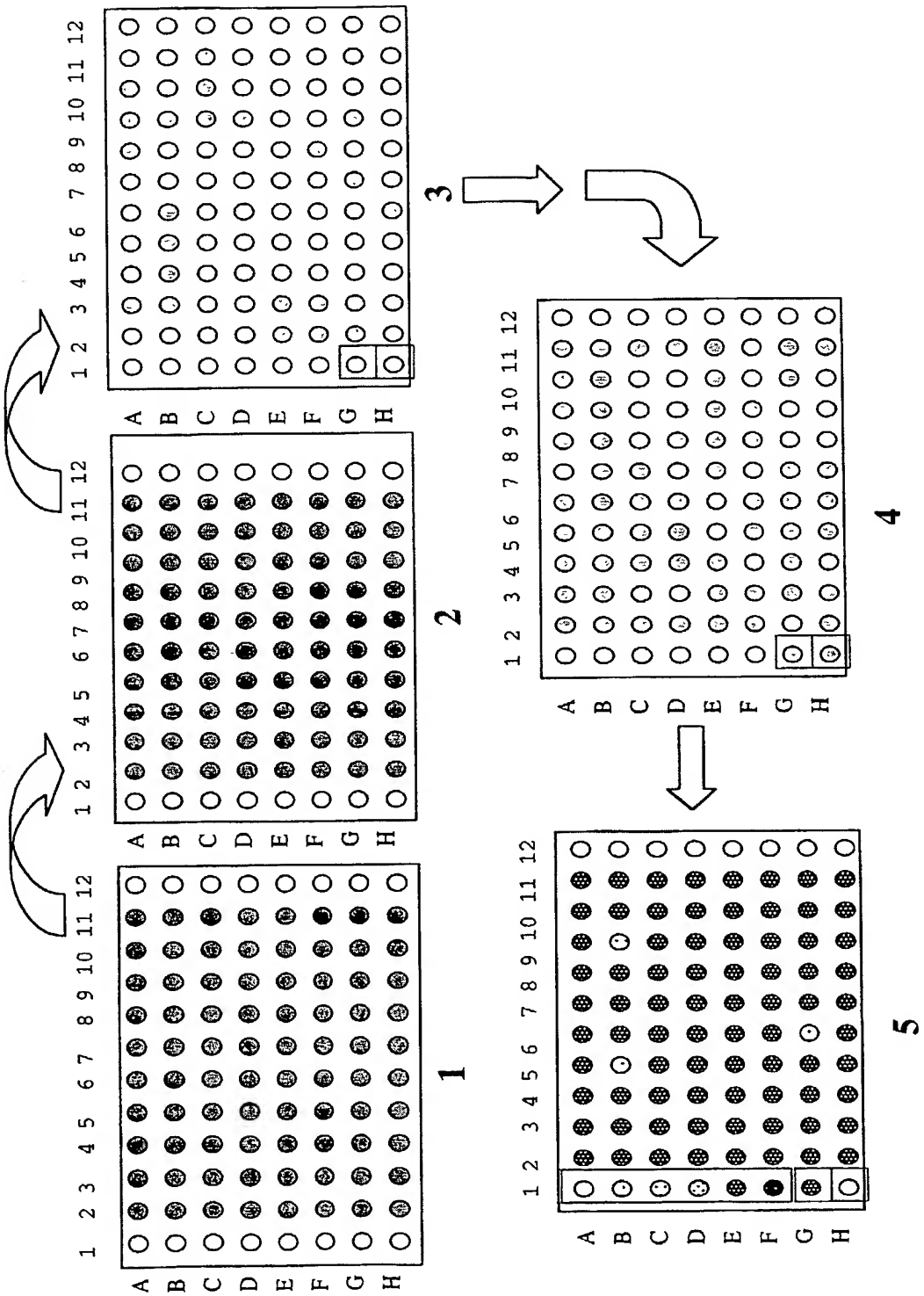
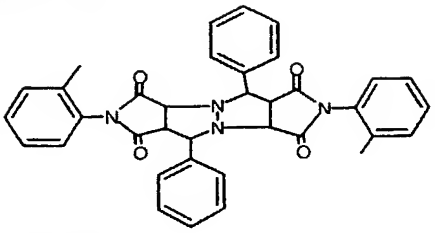
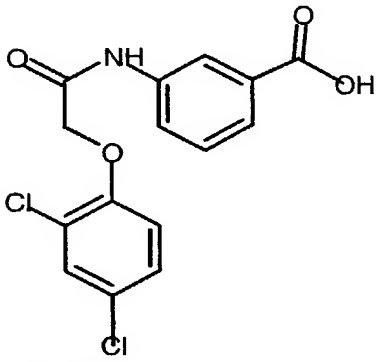


Fig. 16

100764



106160



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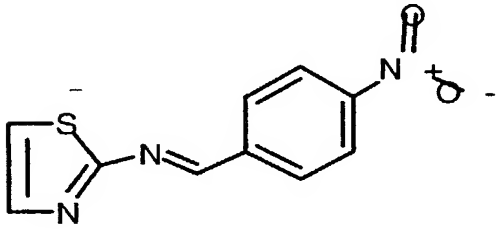

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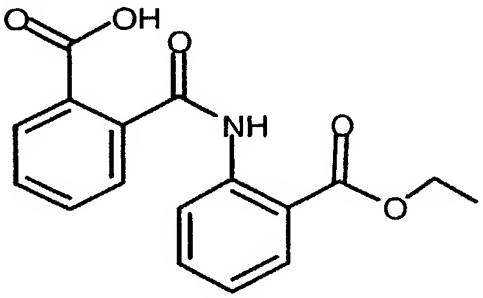
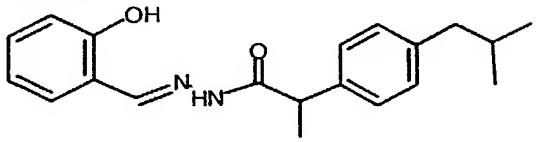
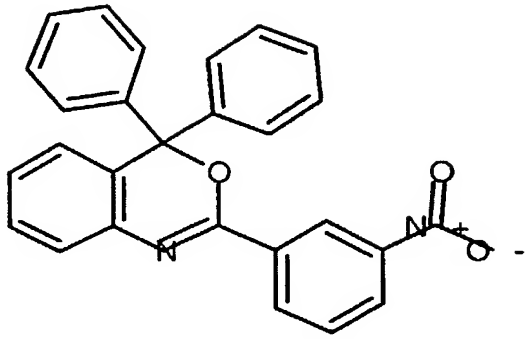


108489



112710



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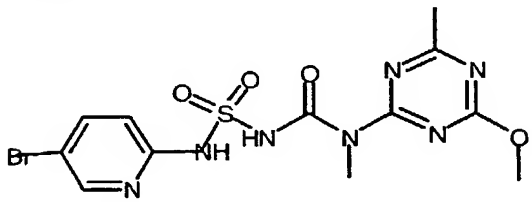

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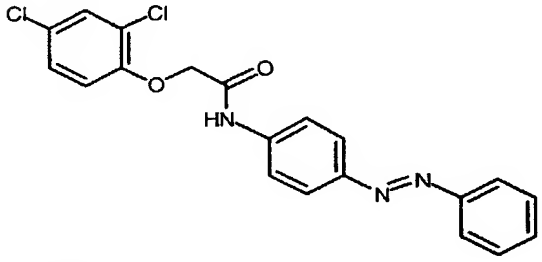

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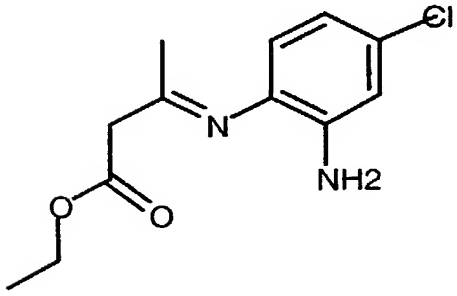
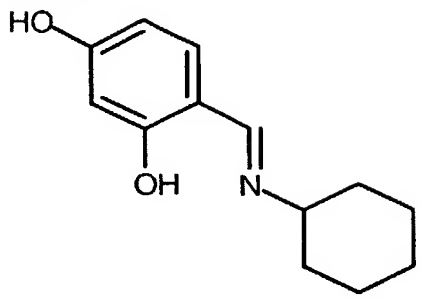


Fig. 17 A

115308



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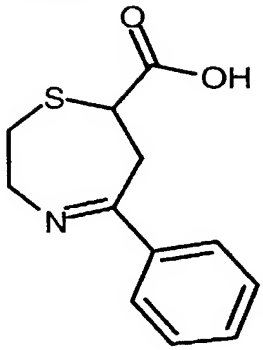

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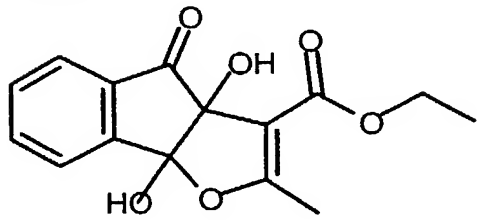

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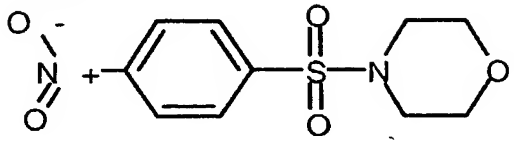

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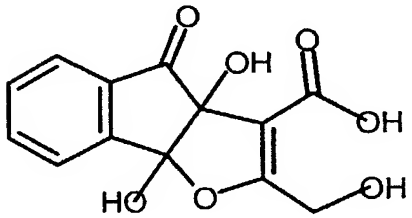

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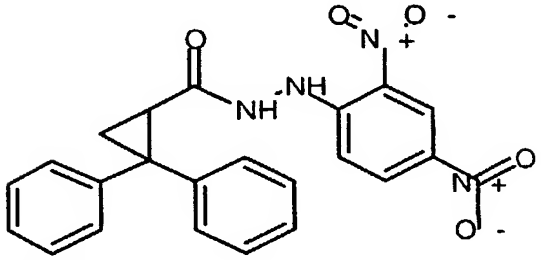

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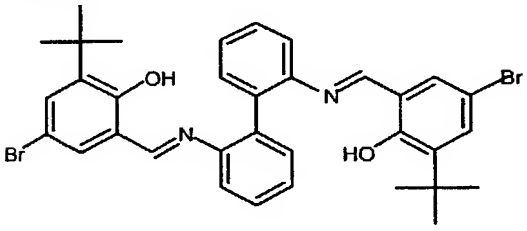

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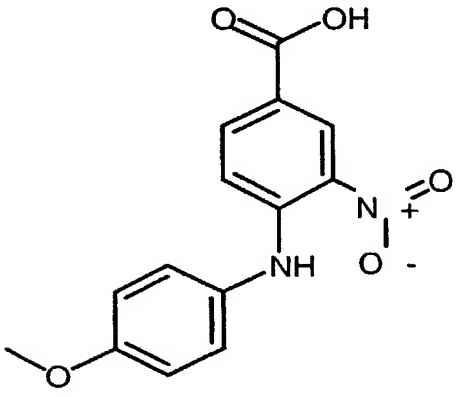

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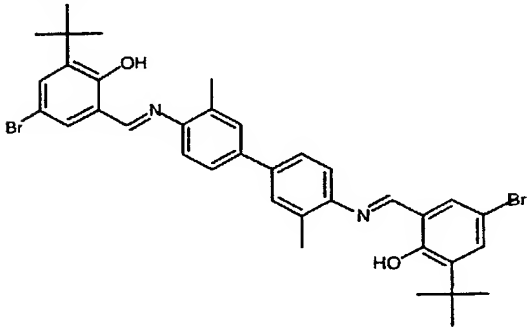
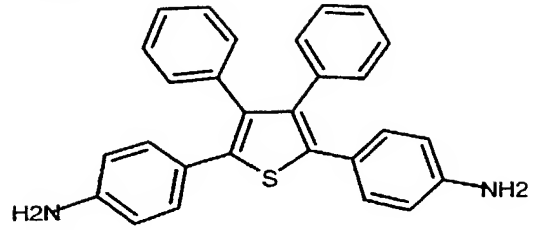


Fig. 17B

128067



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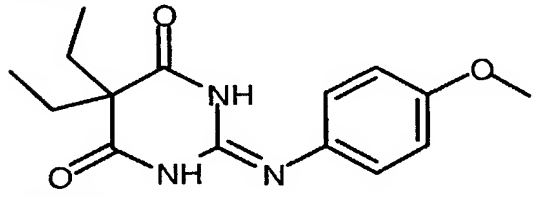

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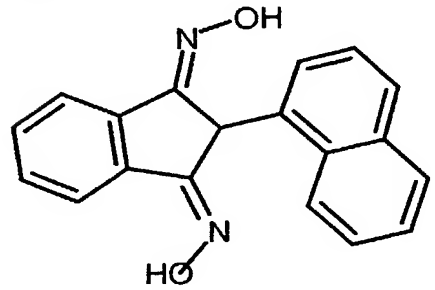

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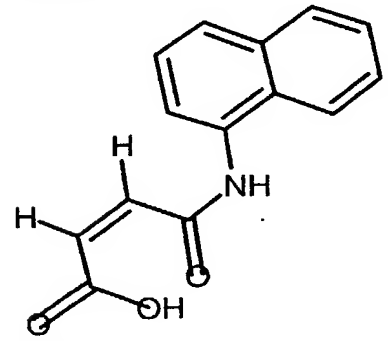

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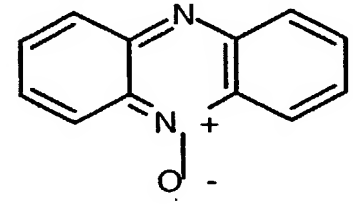

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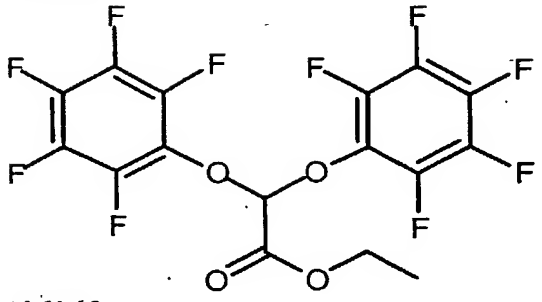

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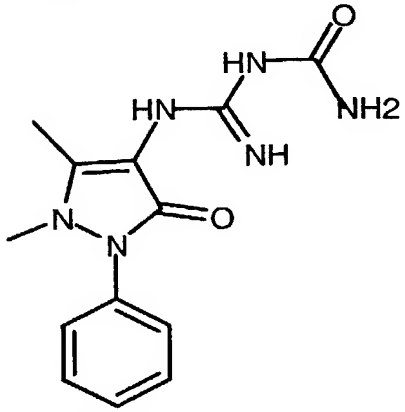

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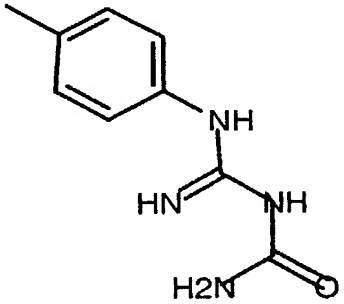

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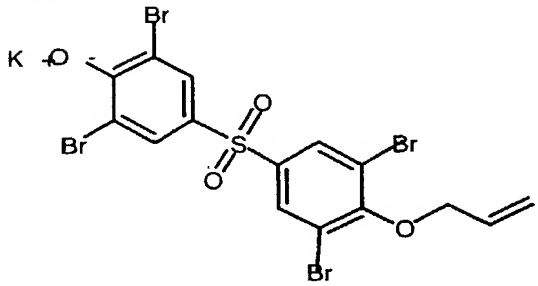
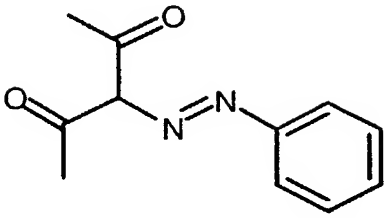


Fig. 17c

140894



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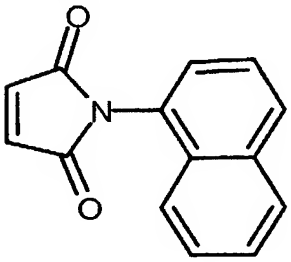

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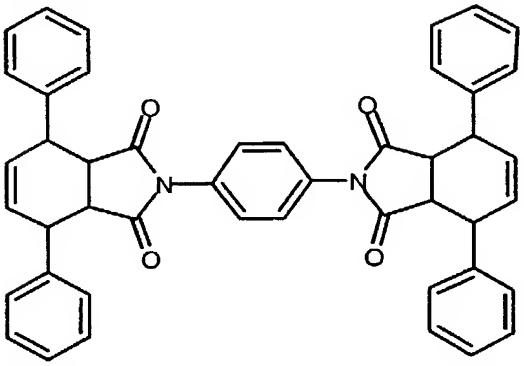

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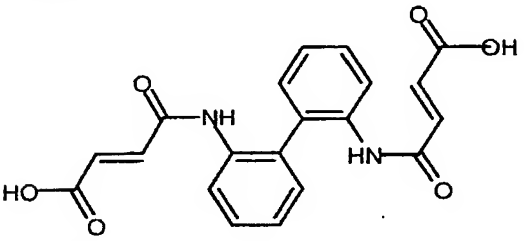

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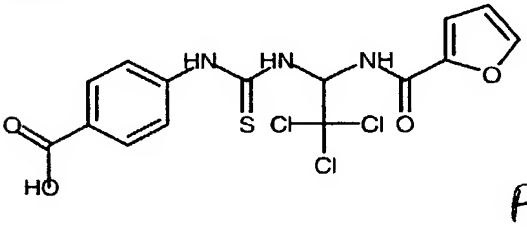

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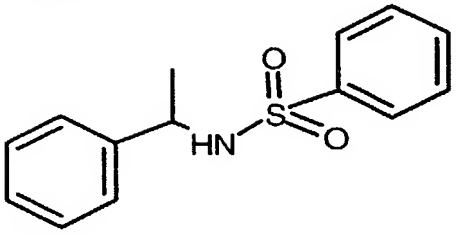

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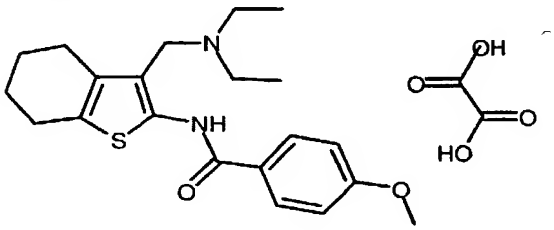

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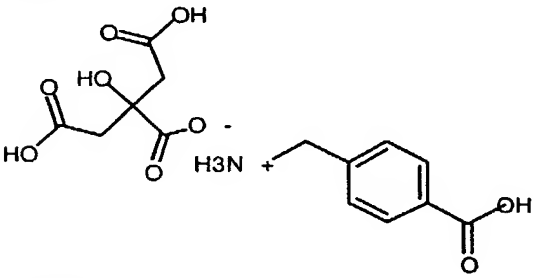

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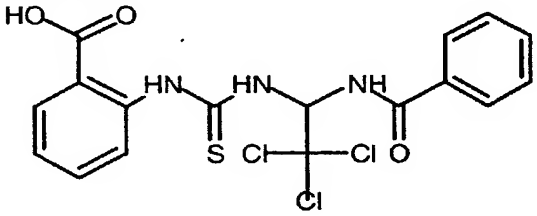

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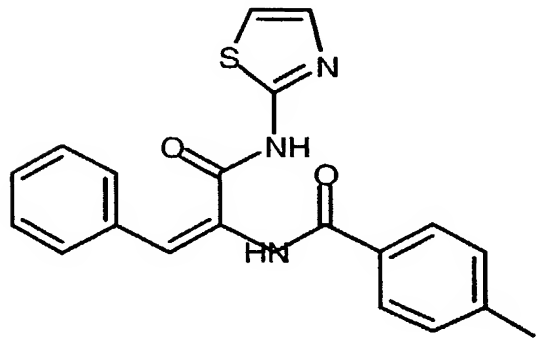

Fig. 17D

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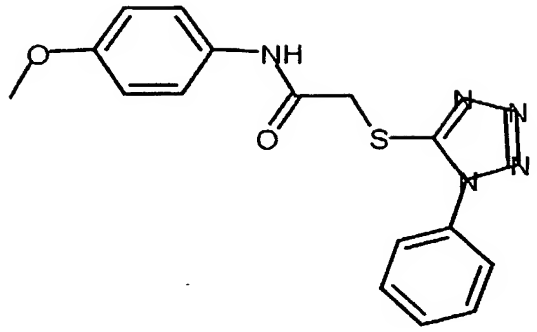

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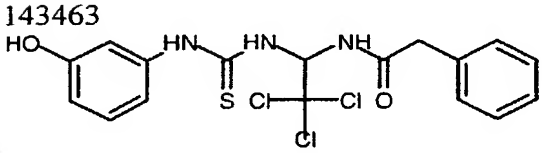

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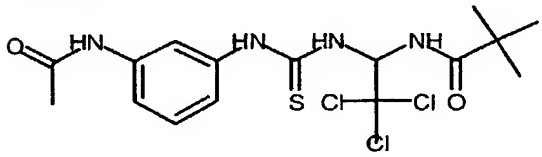

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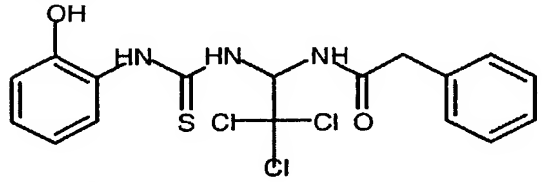

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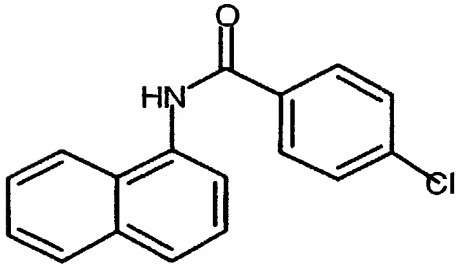

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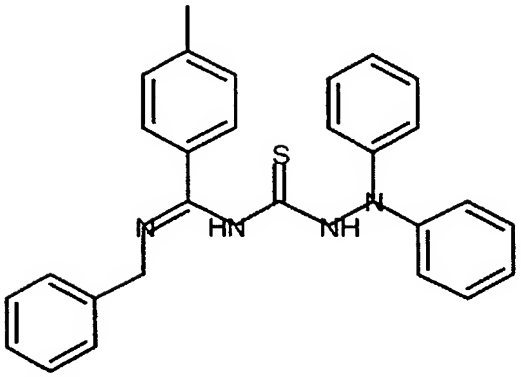

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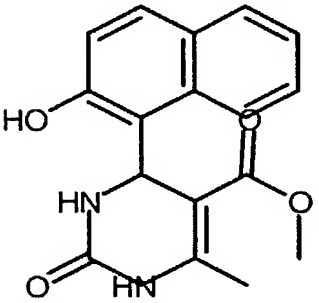

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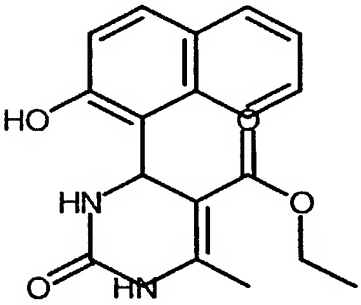

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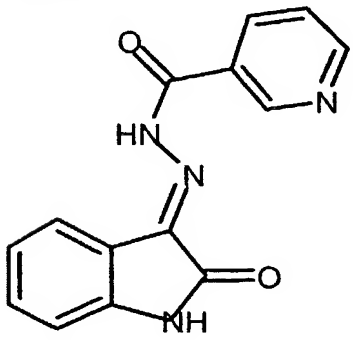

Fig. 17E

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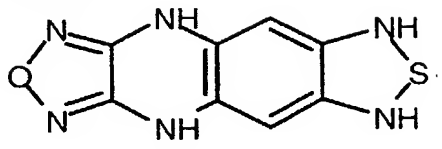

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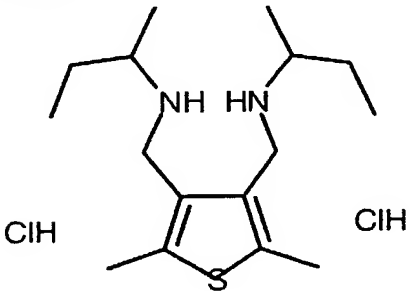

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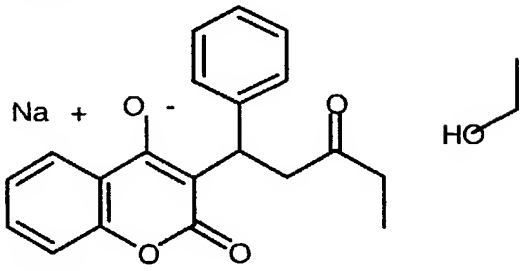

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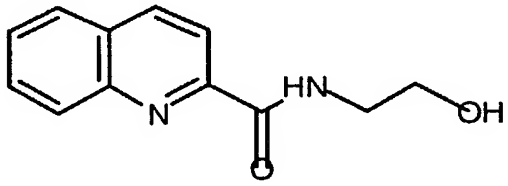

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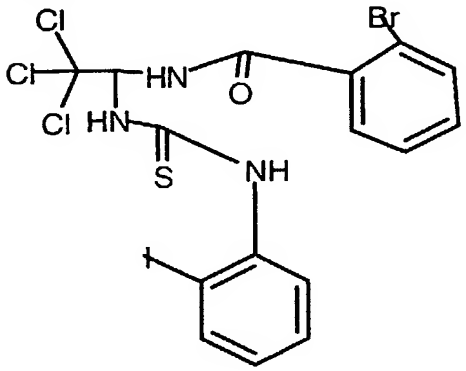

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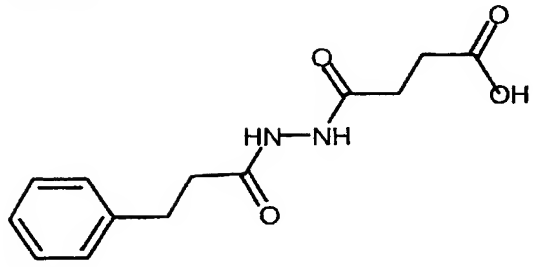

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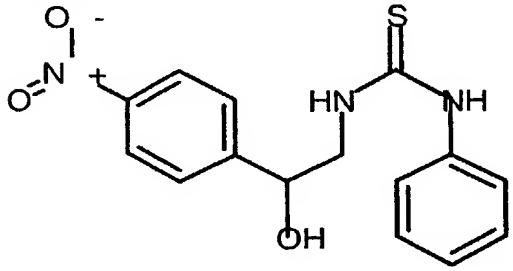

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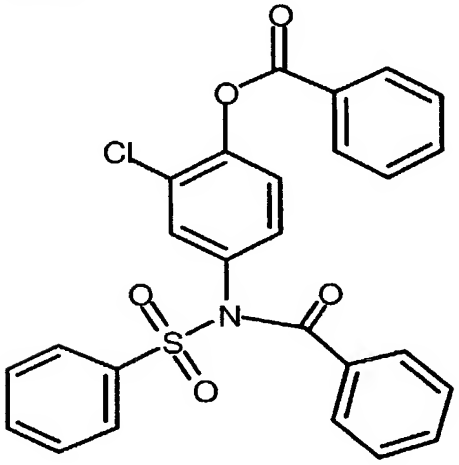

Fig. 17F

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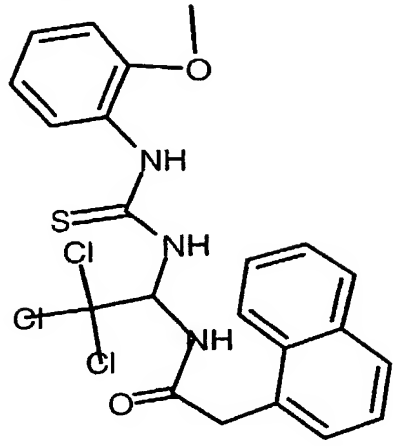

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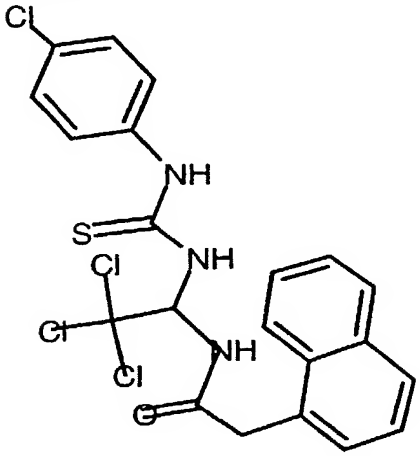

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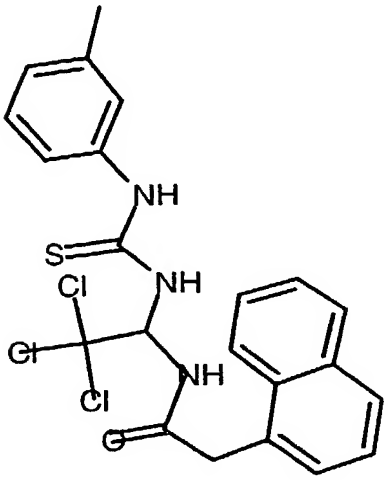

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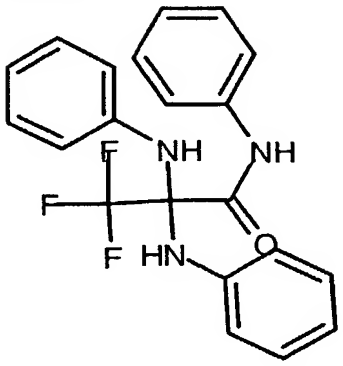

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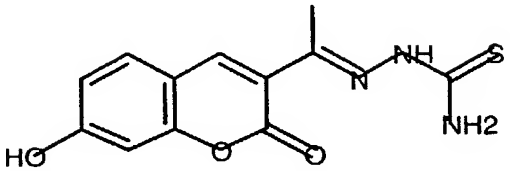

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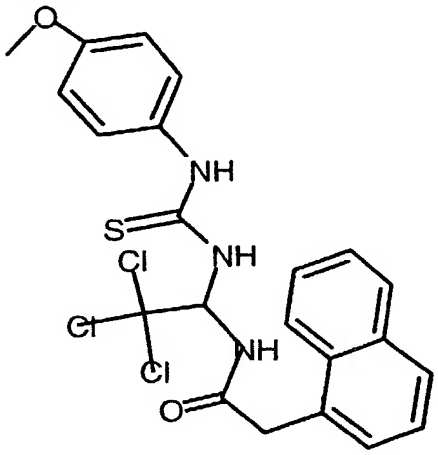

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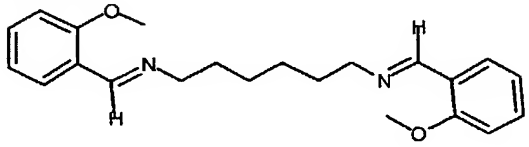

Fig. 17G

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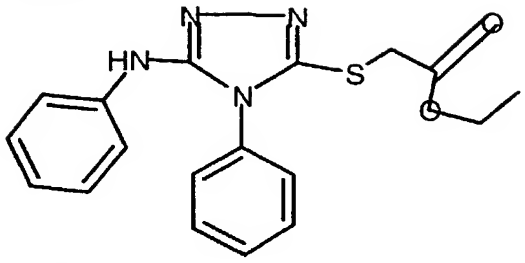

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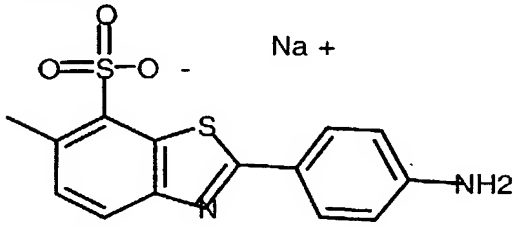

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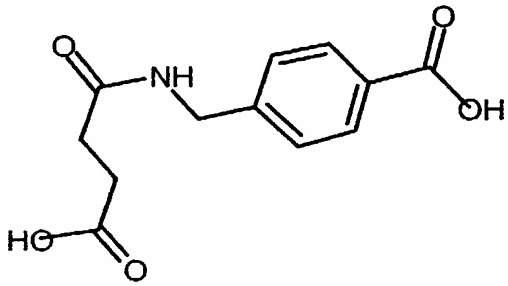

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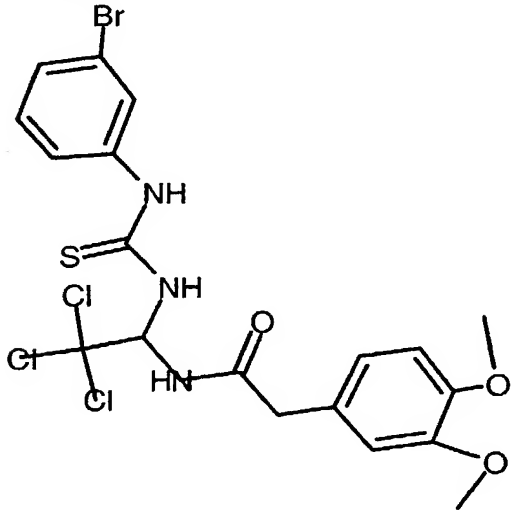

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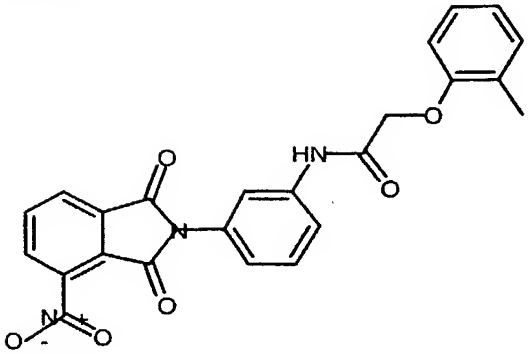

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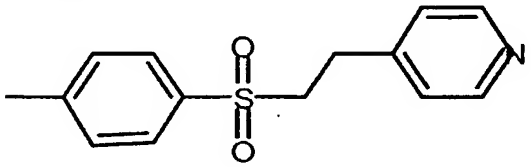

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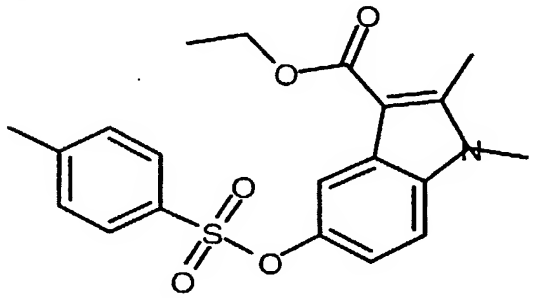

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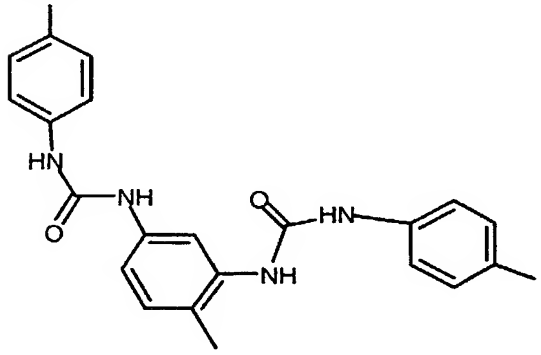

Fig. 17H

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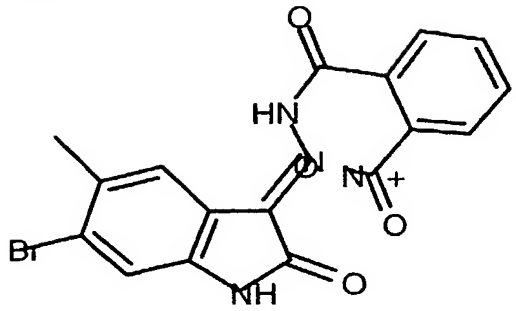

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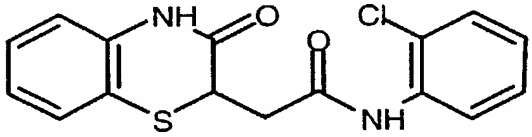

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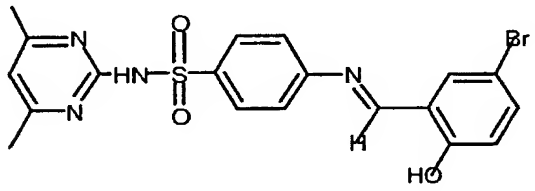

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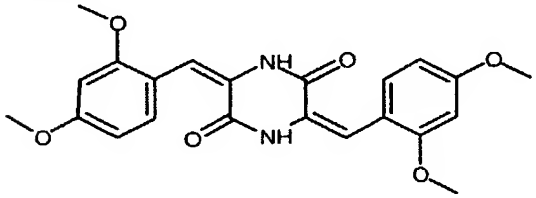

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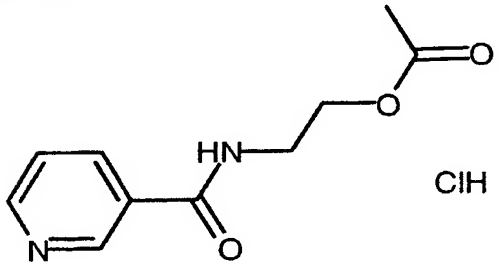

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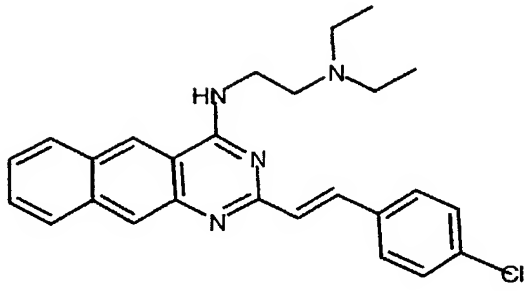

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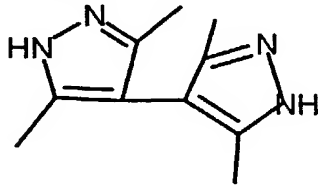

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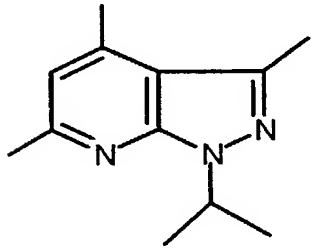

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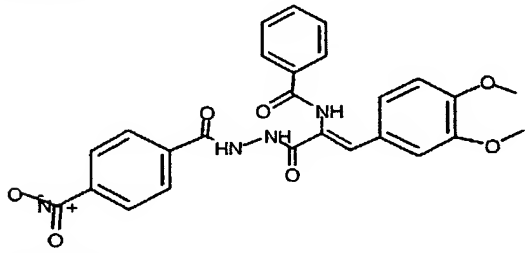

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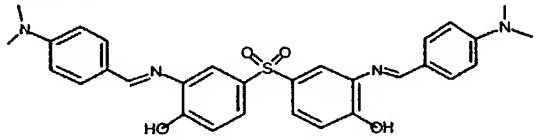

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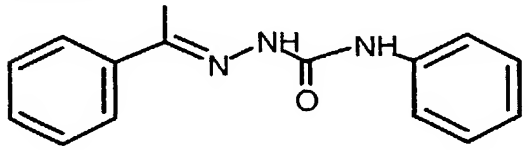
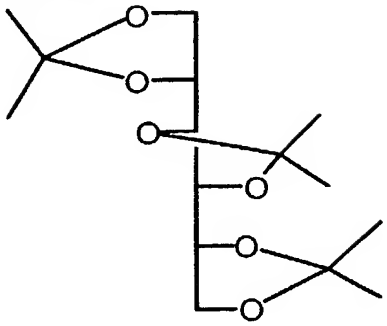


Fig. 17I

107036



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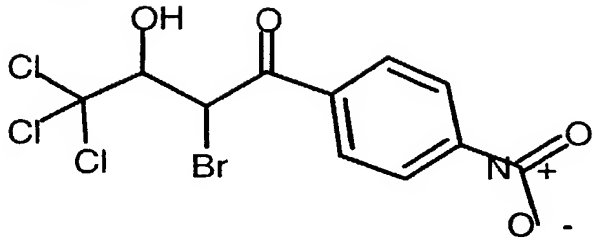

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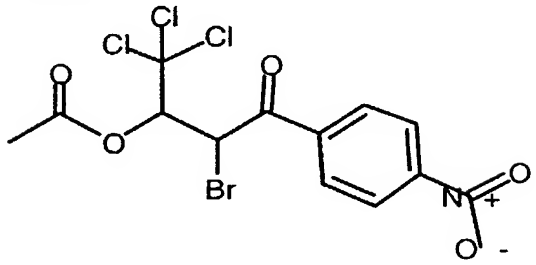

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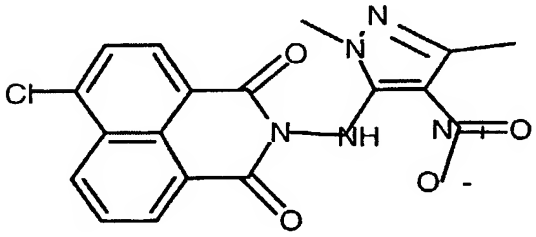

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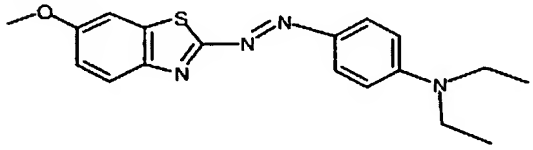

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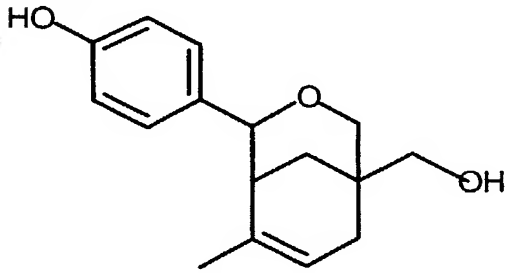

Fig. 17J

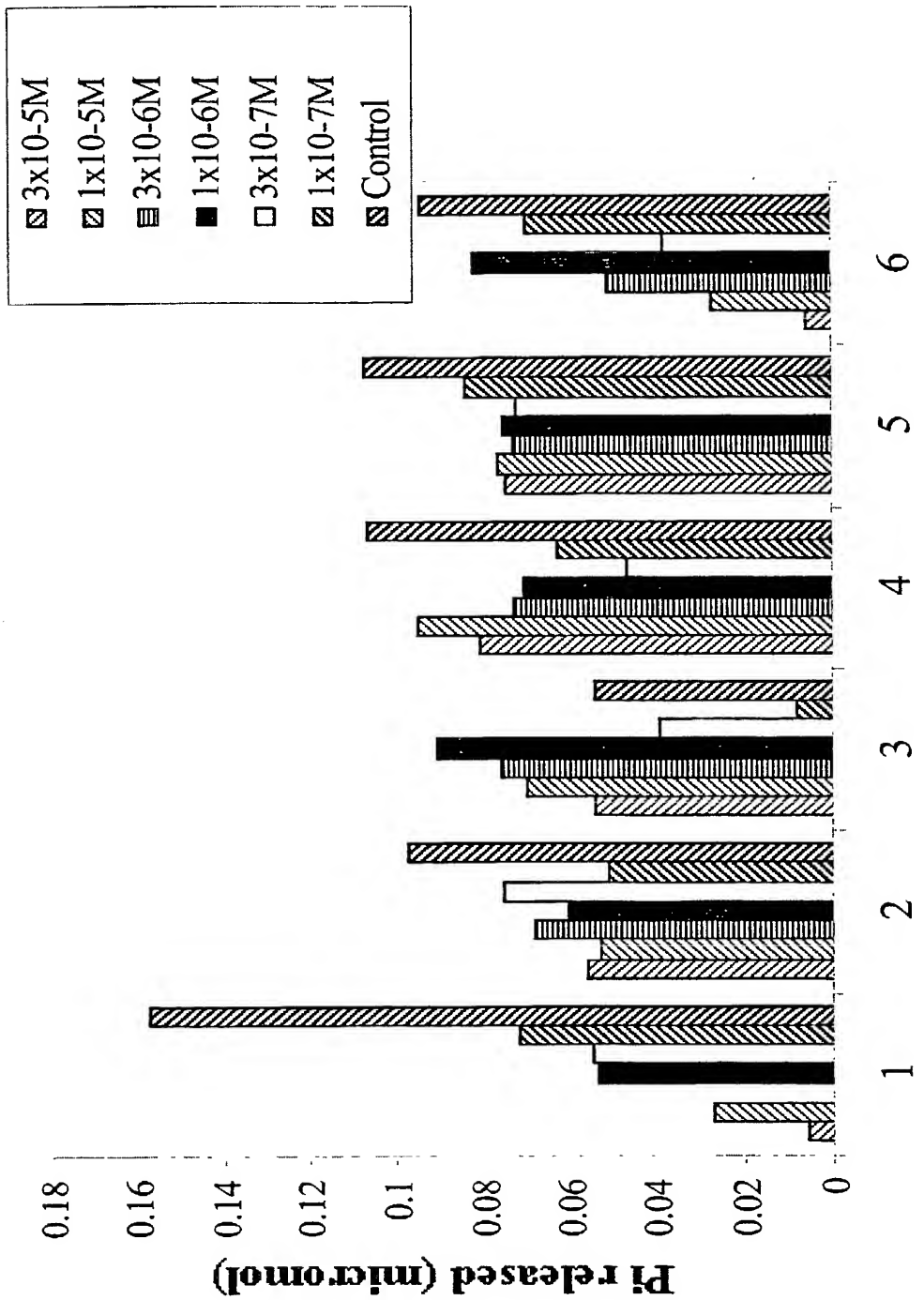
3x10-5M
1x10-5M
3x10-6M
1x10-6M
3x10-7M
1x10-7M
Control
Pi released (micromol)
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
1
2
3
4
5
6

Fig. 18

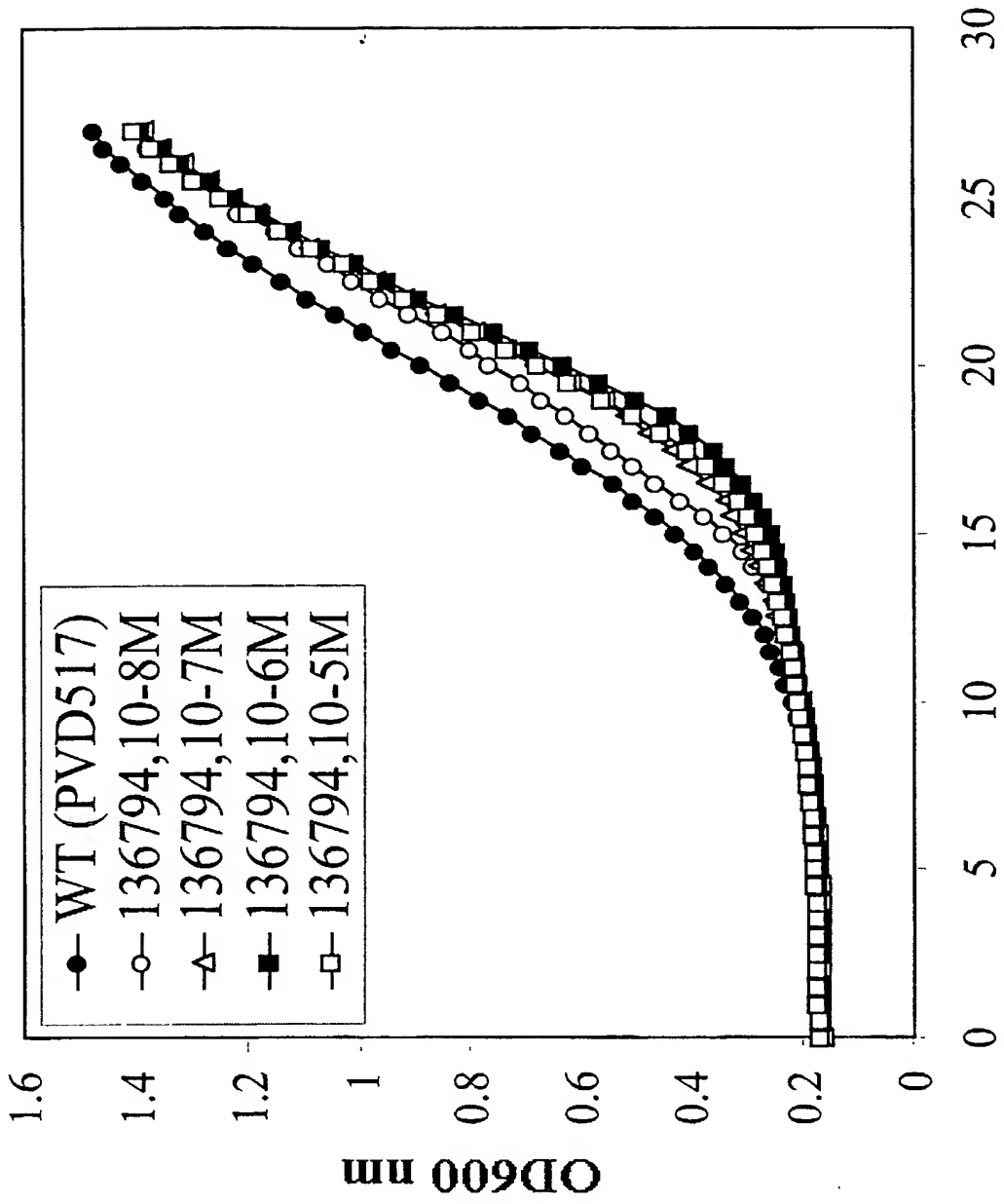
OD600 nm
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
30
Time (hours)
WT (PVD517)
136794,10-8M
136794,10-7M
136794,10-6M
136794,10-5M

Fig. 19A

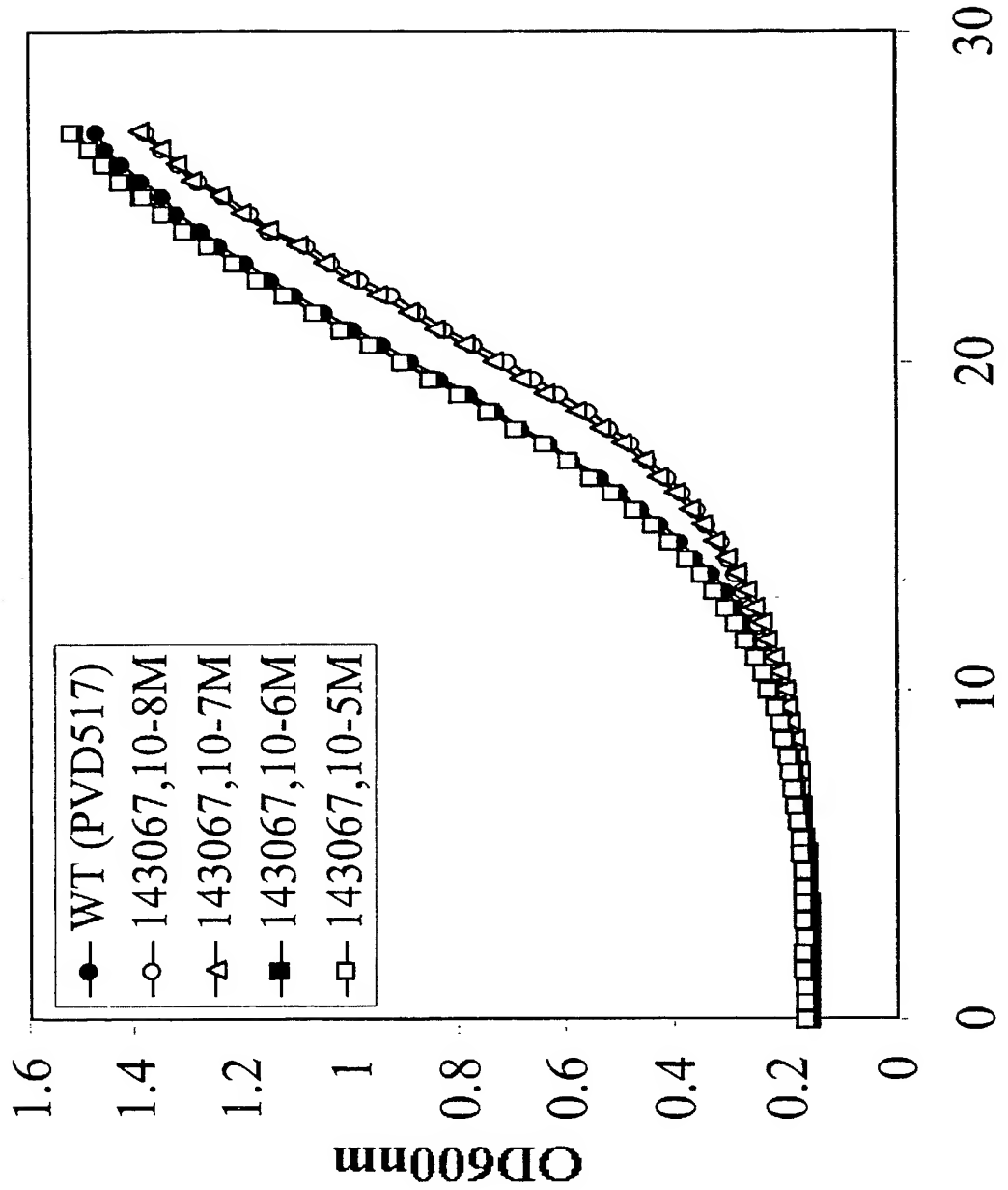
OD600nm
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
Time (hours)
WT (PVD517)
143067,10-8M
143067,10-7M
143067,10-6M
143067,10-5M

Fig. 19B

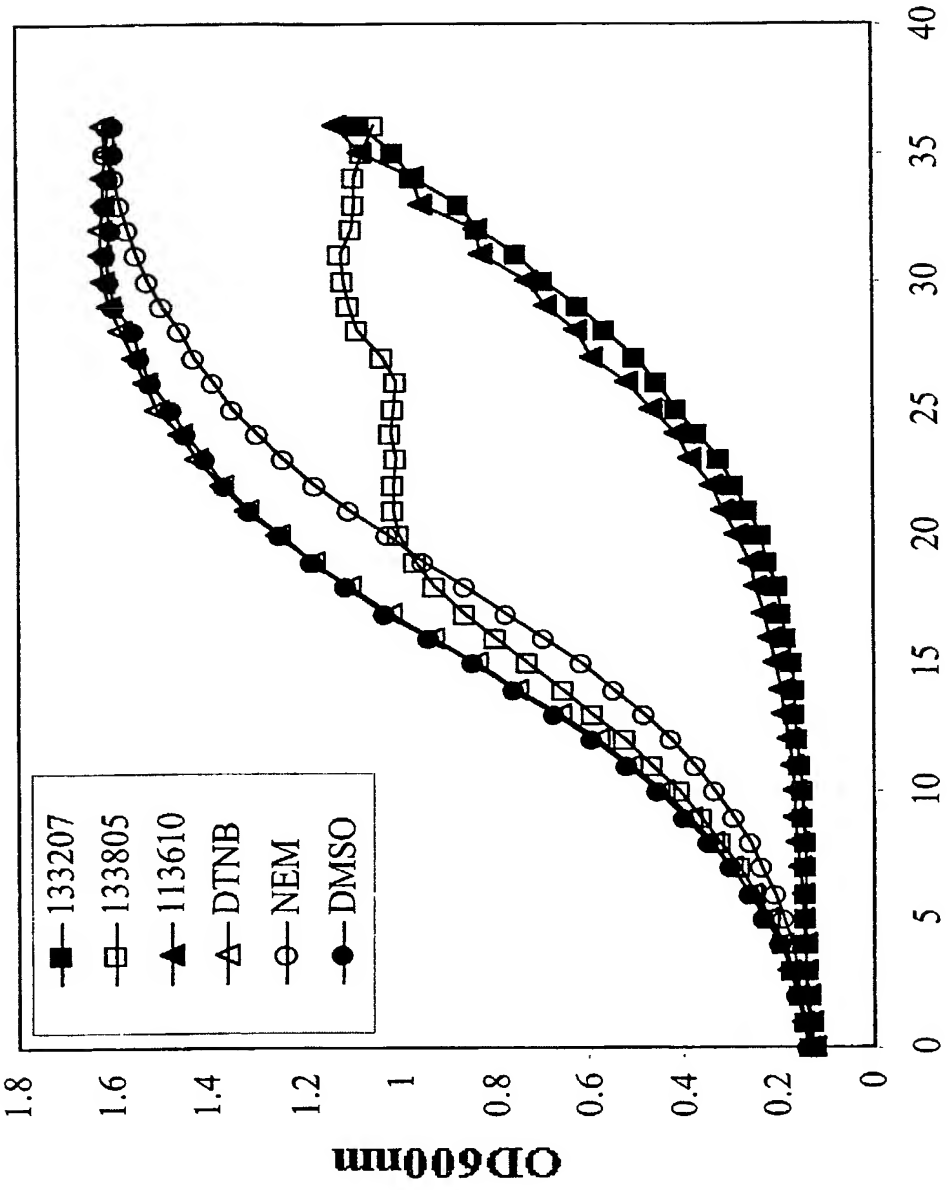
OD600nm
Time (hours)
133207
133805
113610
DTNB
NEM
DMSO
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
0
5
10
15
20
25
30
35
40

Fig. 20A

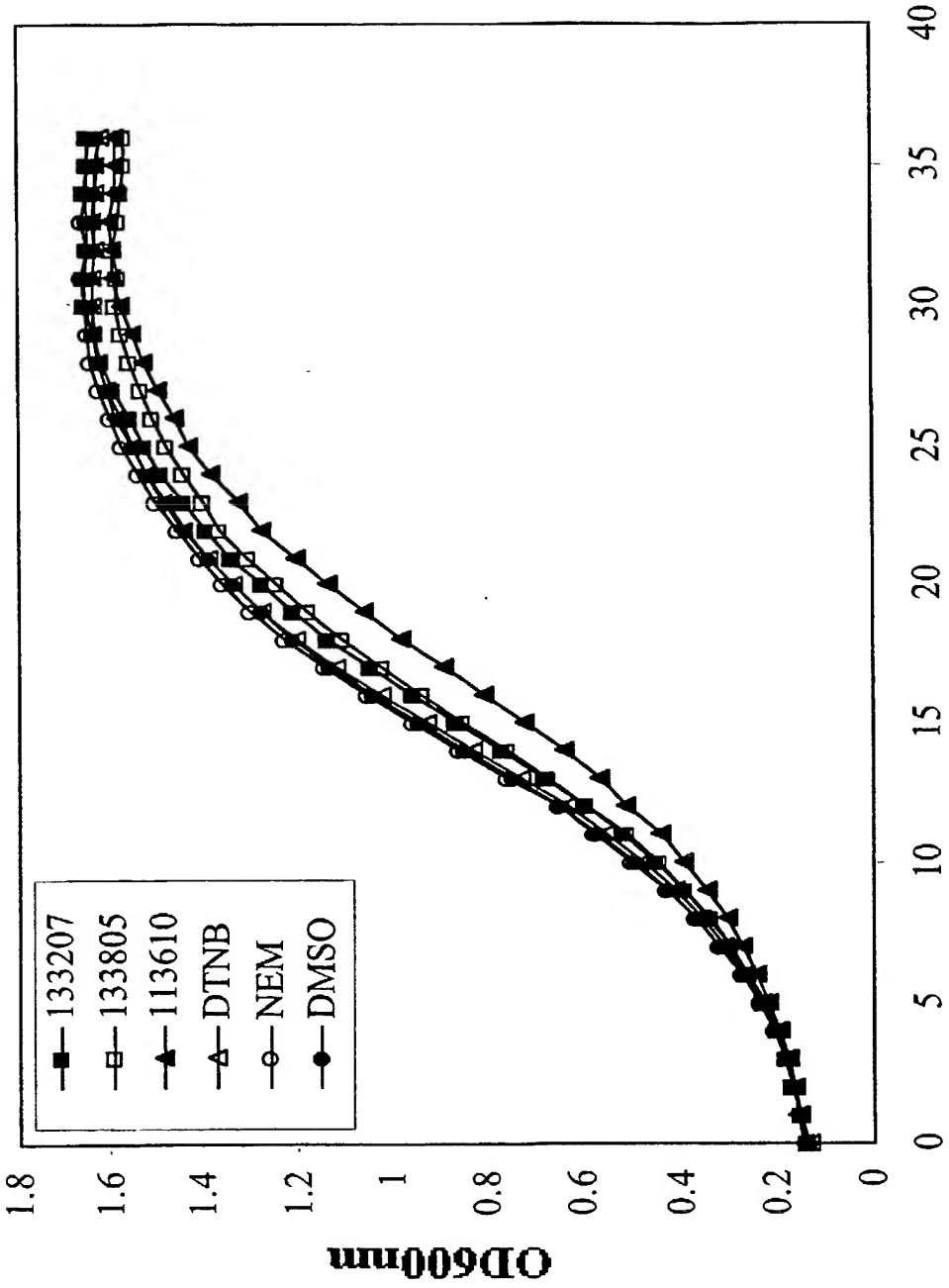
OD600nm
Time (hours)
133207
133805
113610
DTNB
NEM
DMSO
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
0
5
10
15
20
25
30
35
40

Fig. 20B

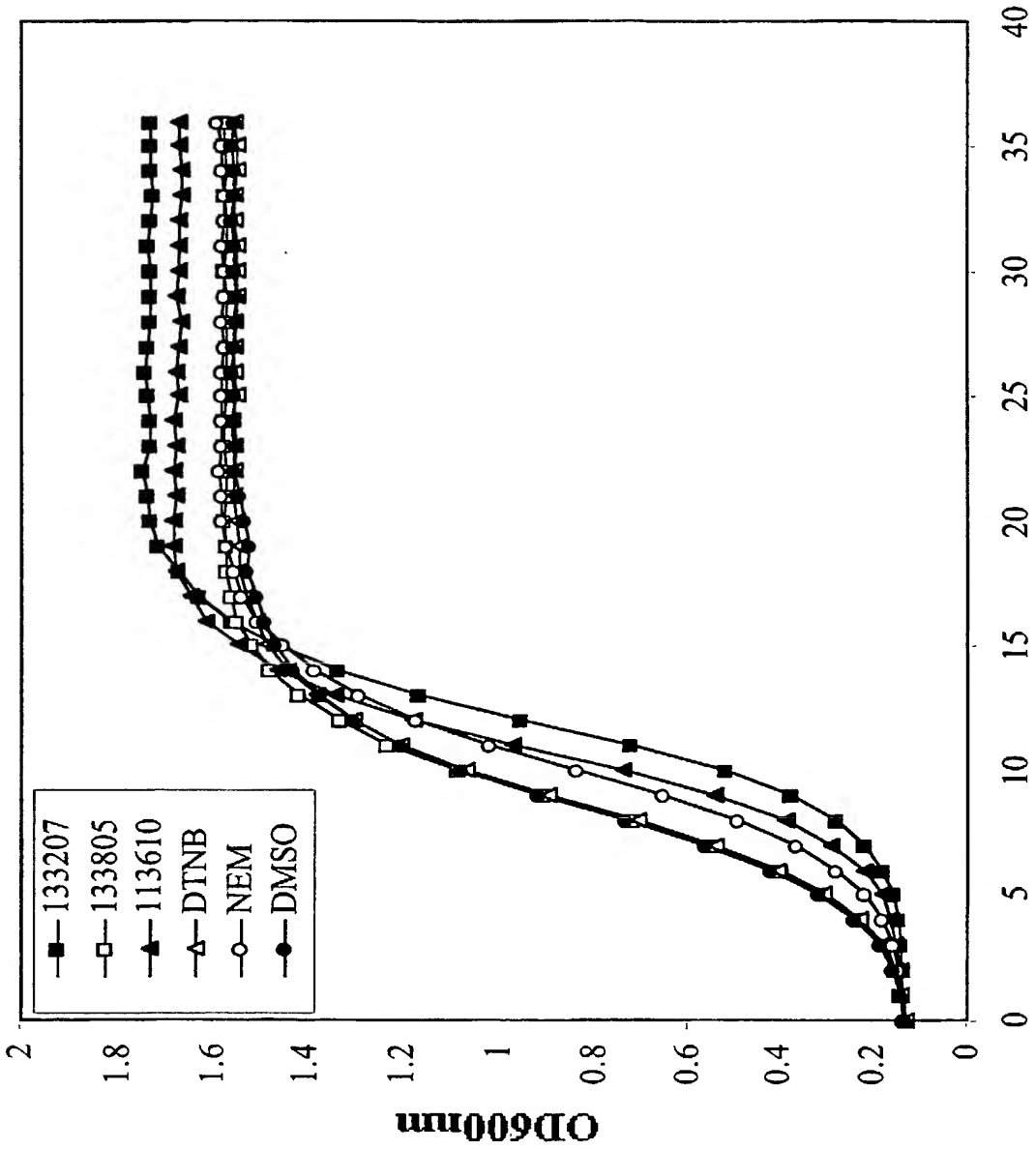
OD600nm
Time (hours)
133207
133805
113610
DTNB
NEM
DMSO
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
0
5
10
15
20
25
30
35
40

Fig. 21A

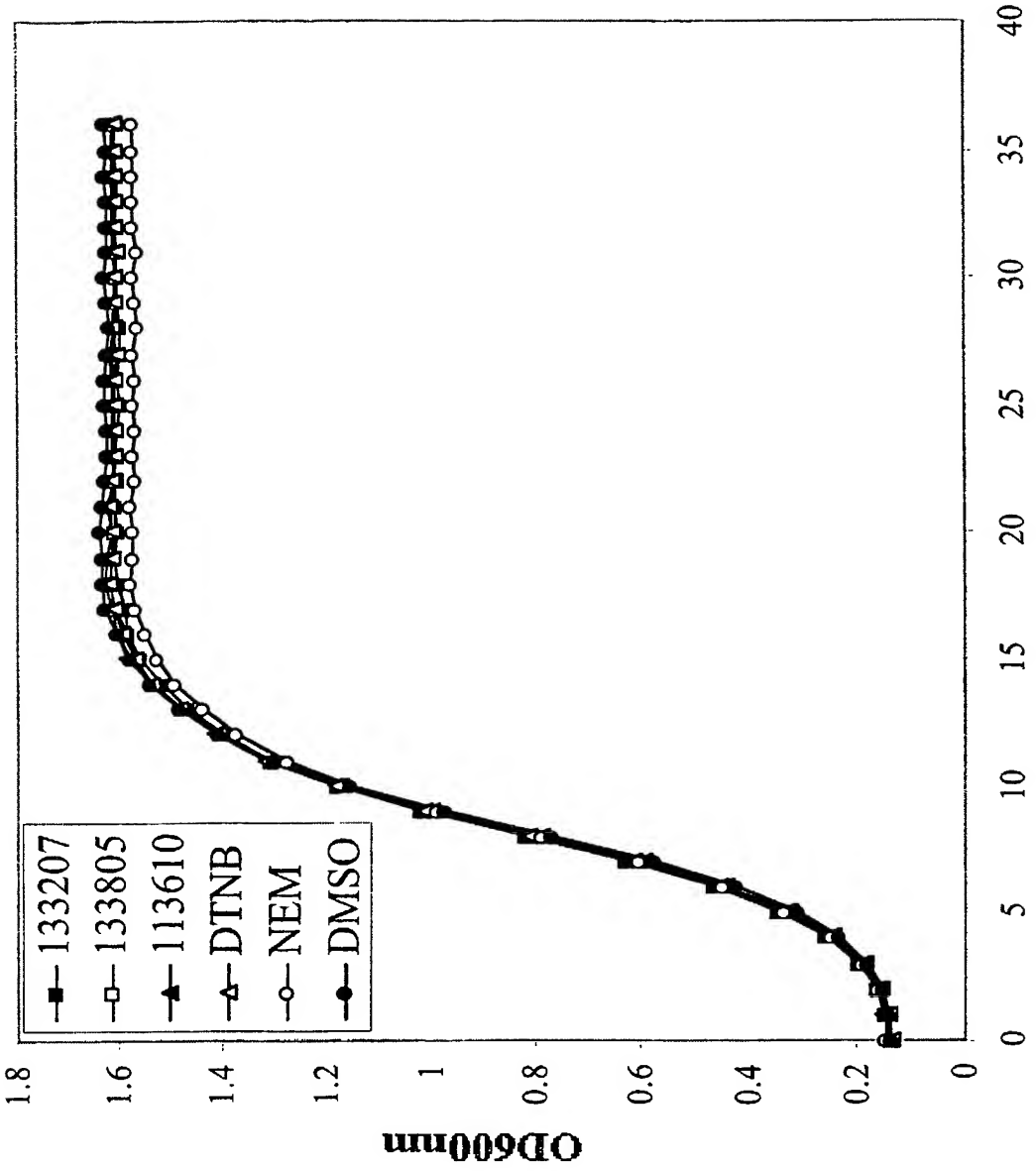
OD600nm
Time (hours)
133207
133805
113610
DTNB
NEM
DMSO
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
0
5
10
15
20
25
30
35
40

Fig. 21B

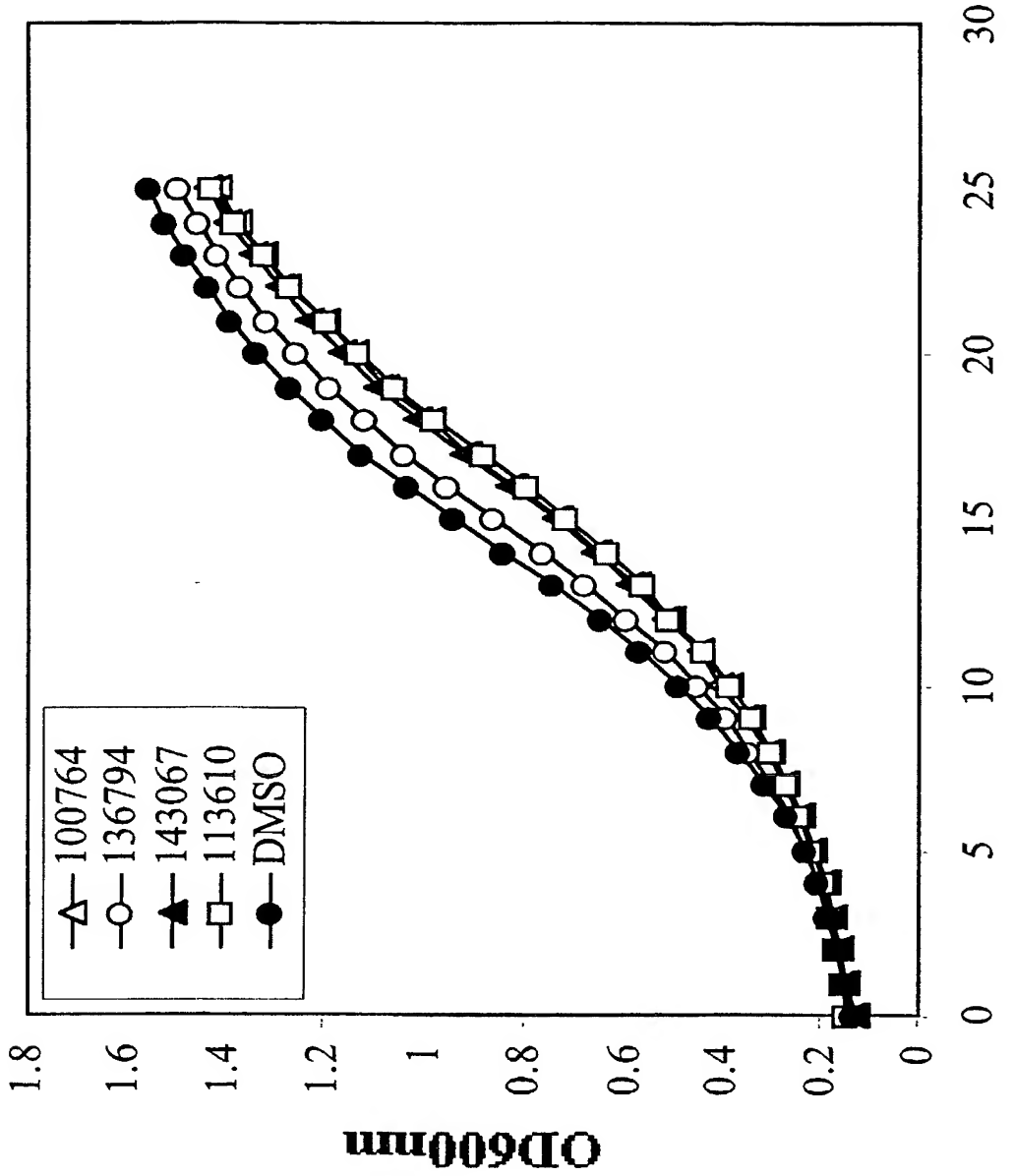
OD600nm
Time (hours)
100764
136794
143067
113610
DMSO
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
0
5
10
15
20
25
30

Fig. 22

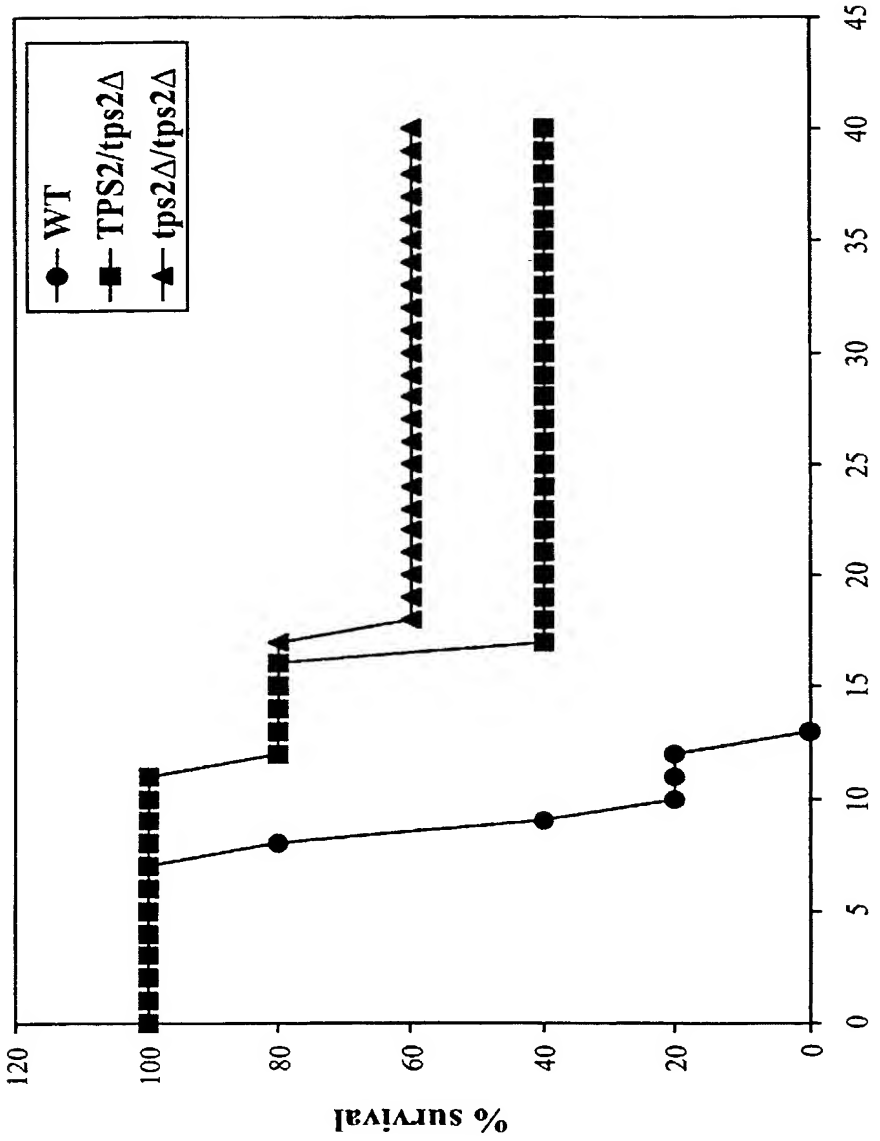
WT
TPS2/tps2Δ
tps2Δ/tps2Δ
% survival
120
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
45
Time after infection (days)

Fig. 23A

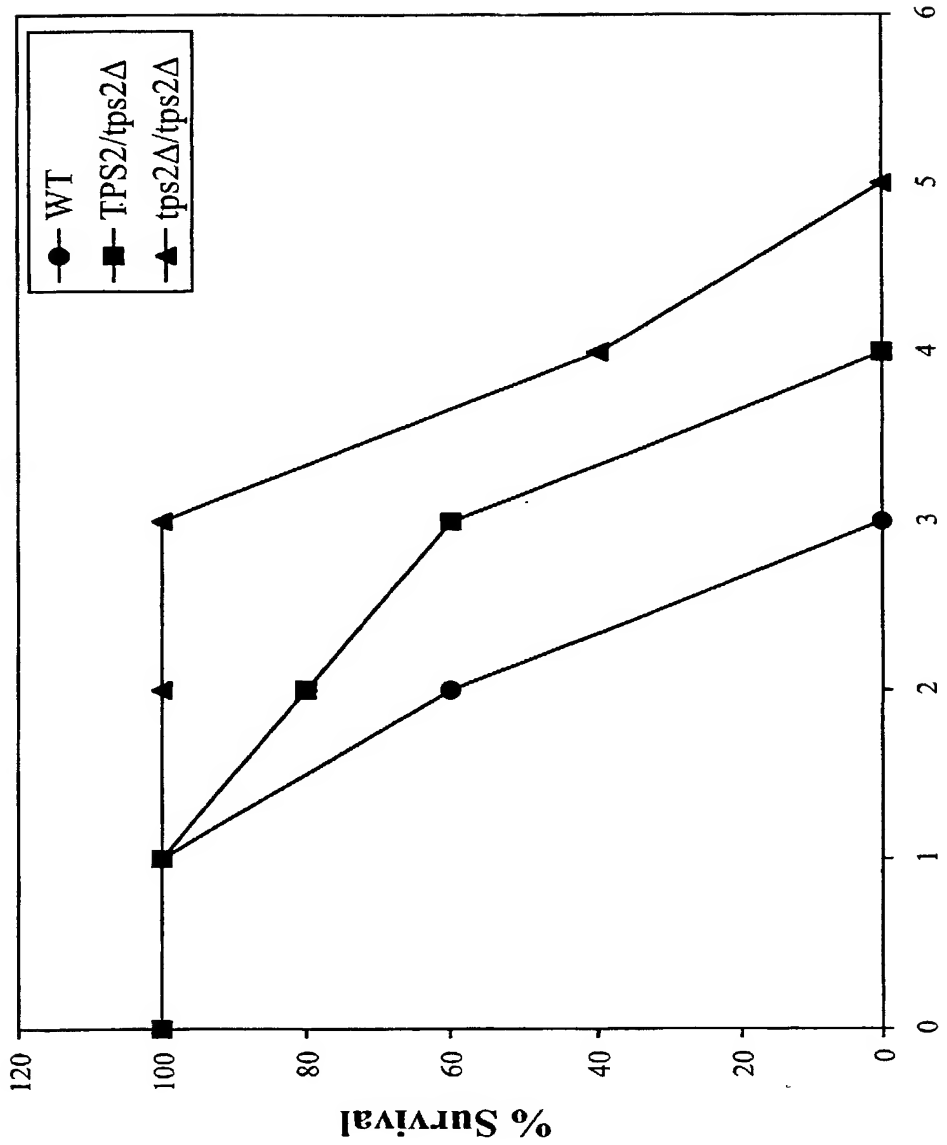
WT
TPS2/tps2Δ
tps2Δ/tps2Δ
% Survival
120
100
80
60
40
20
0
0
1
2
3
4
5
6
Time after infection (days)

Fig. 23B

The *C. albicans tps2Δ/tps2Δ* strain is sensitive to heat stress at 44 °C

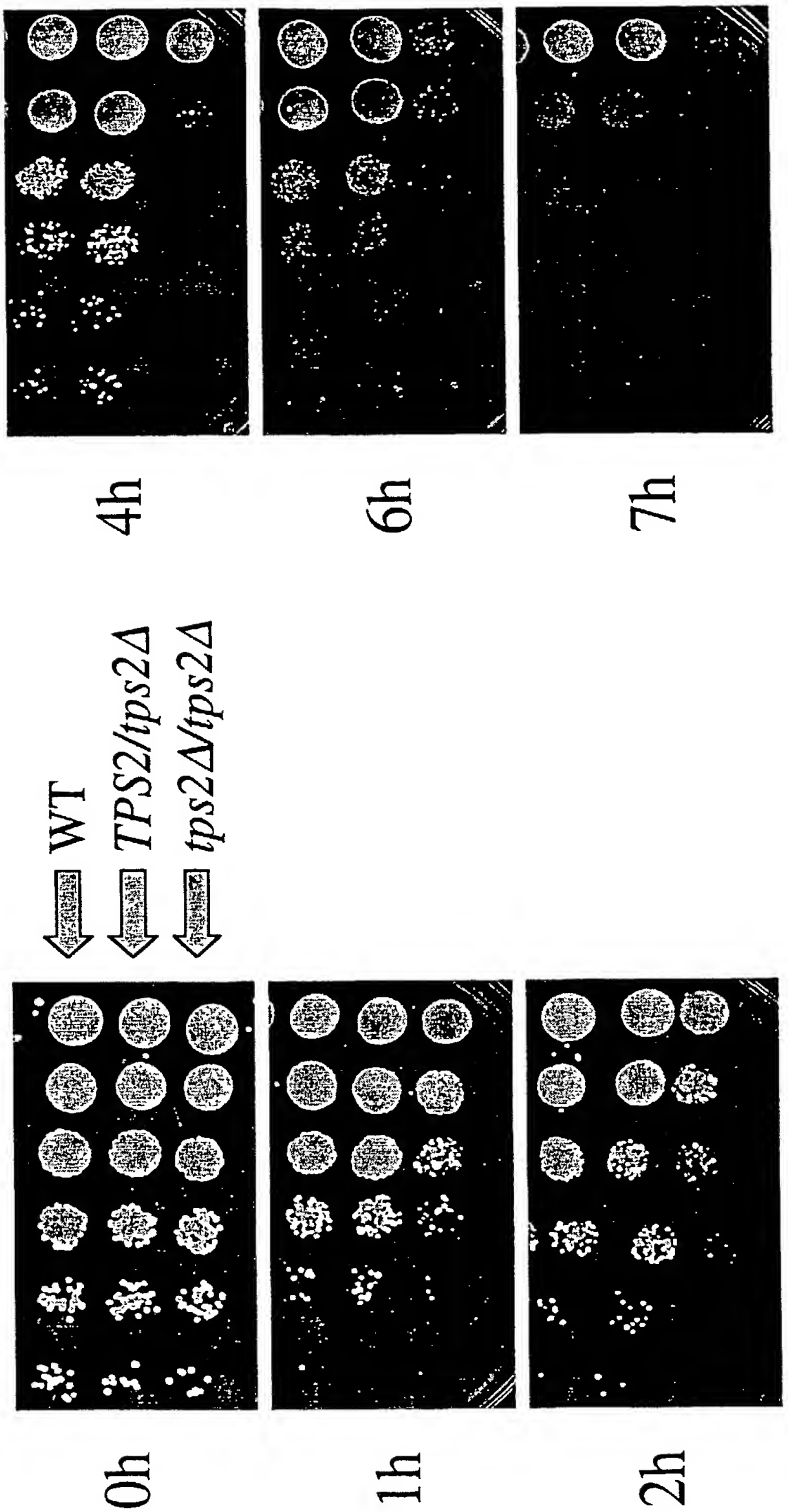

Fig. 24

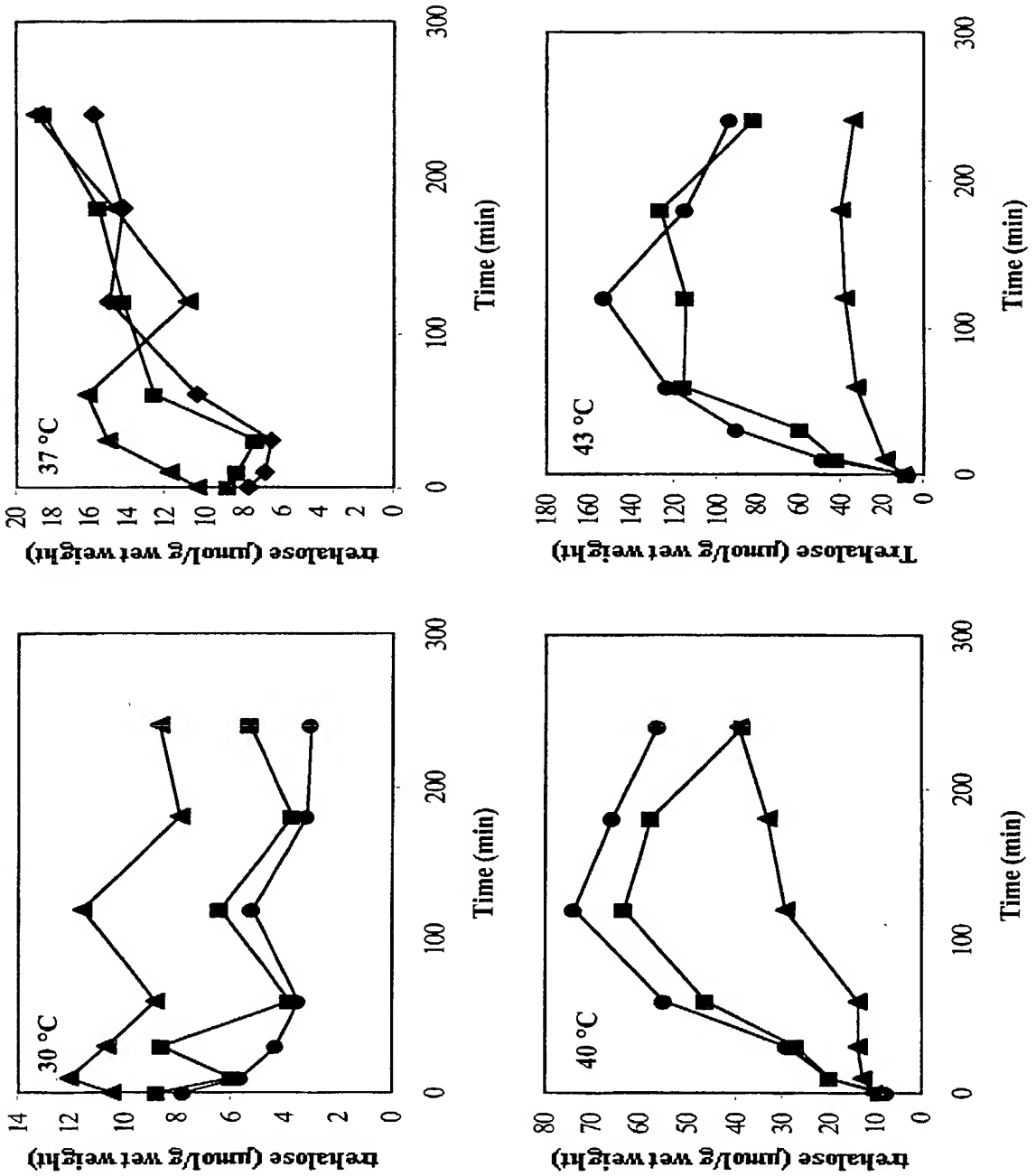

Fig. 25

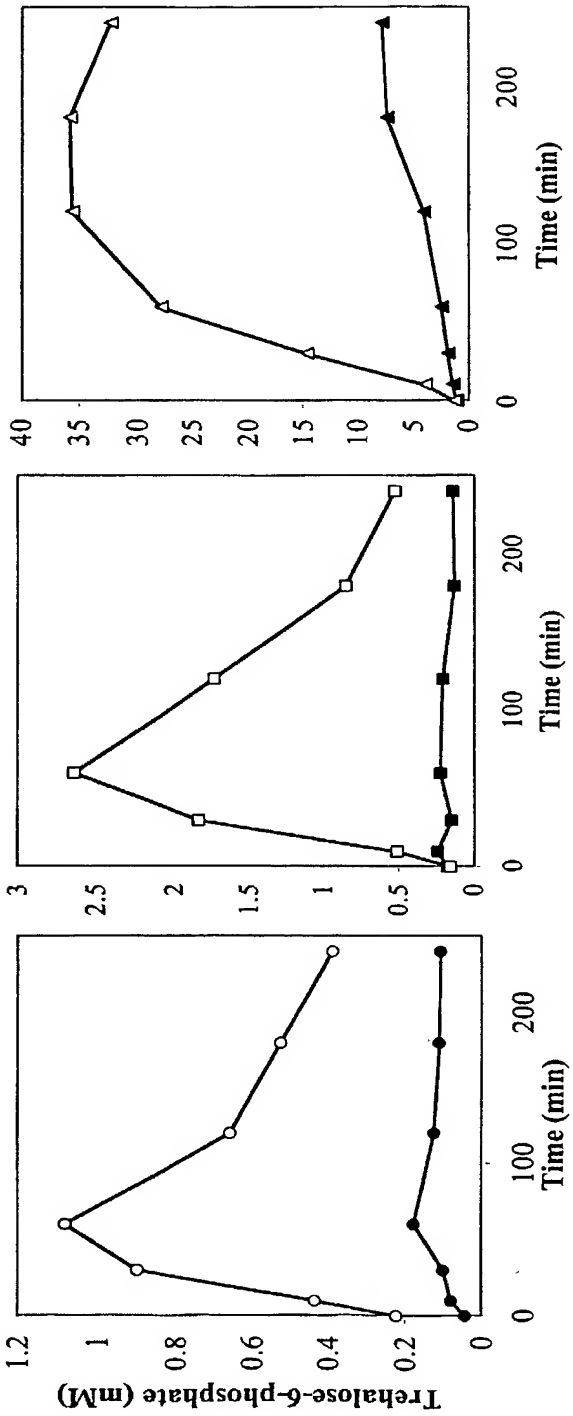

Fig. 26

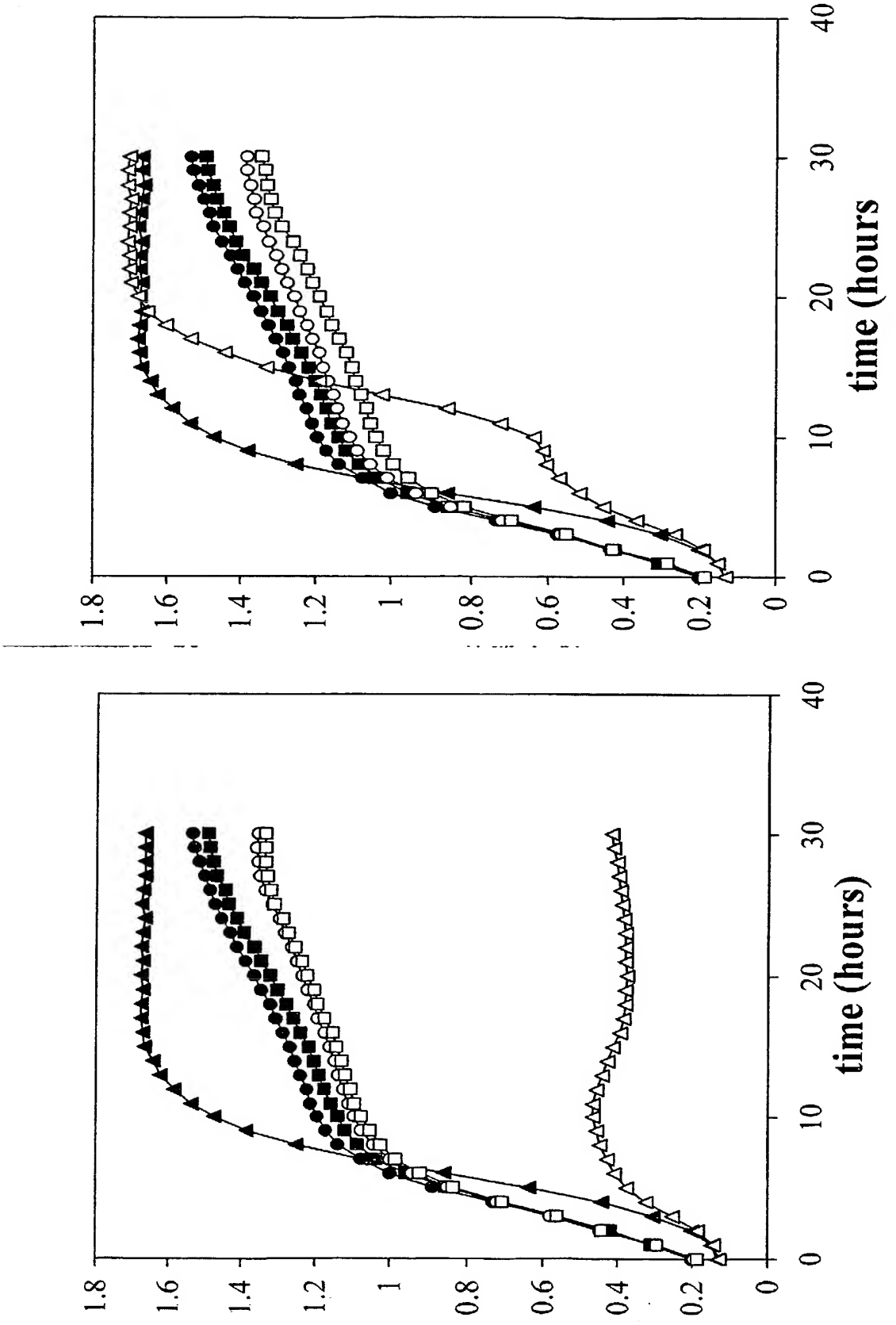
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
time (hours
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
time (hours)

Fig. 27

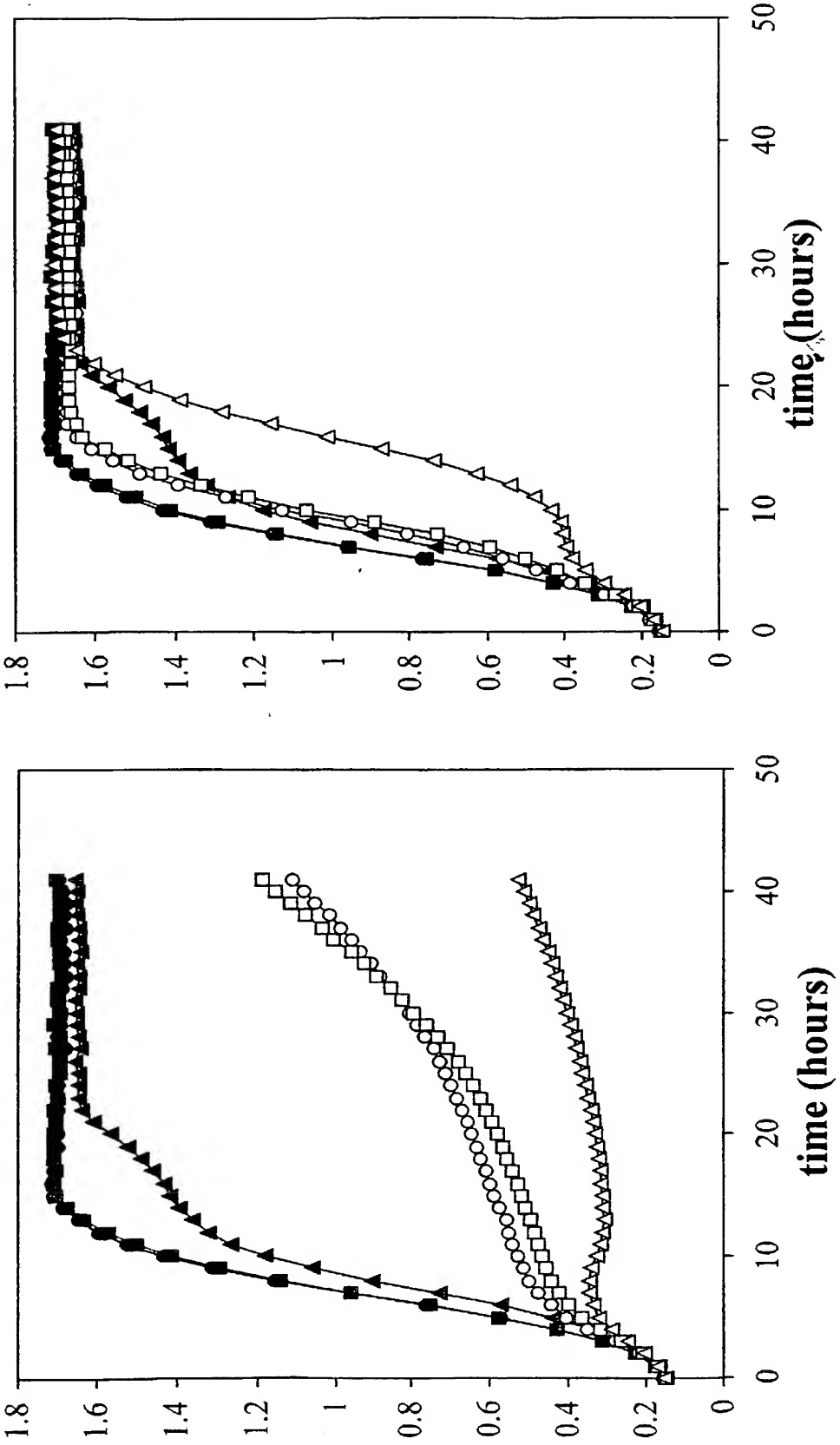
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
time (hours)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
time (hours)

Fig. 28

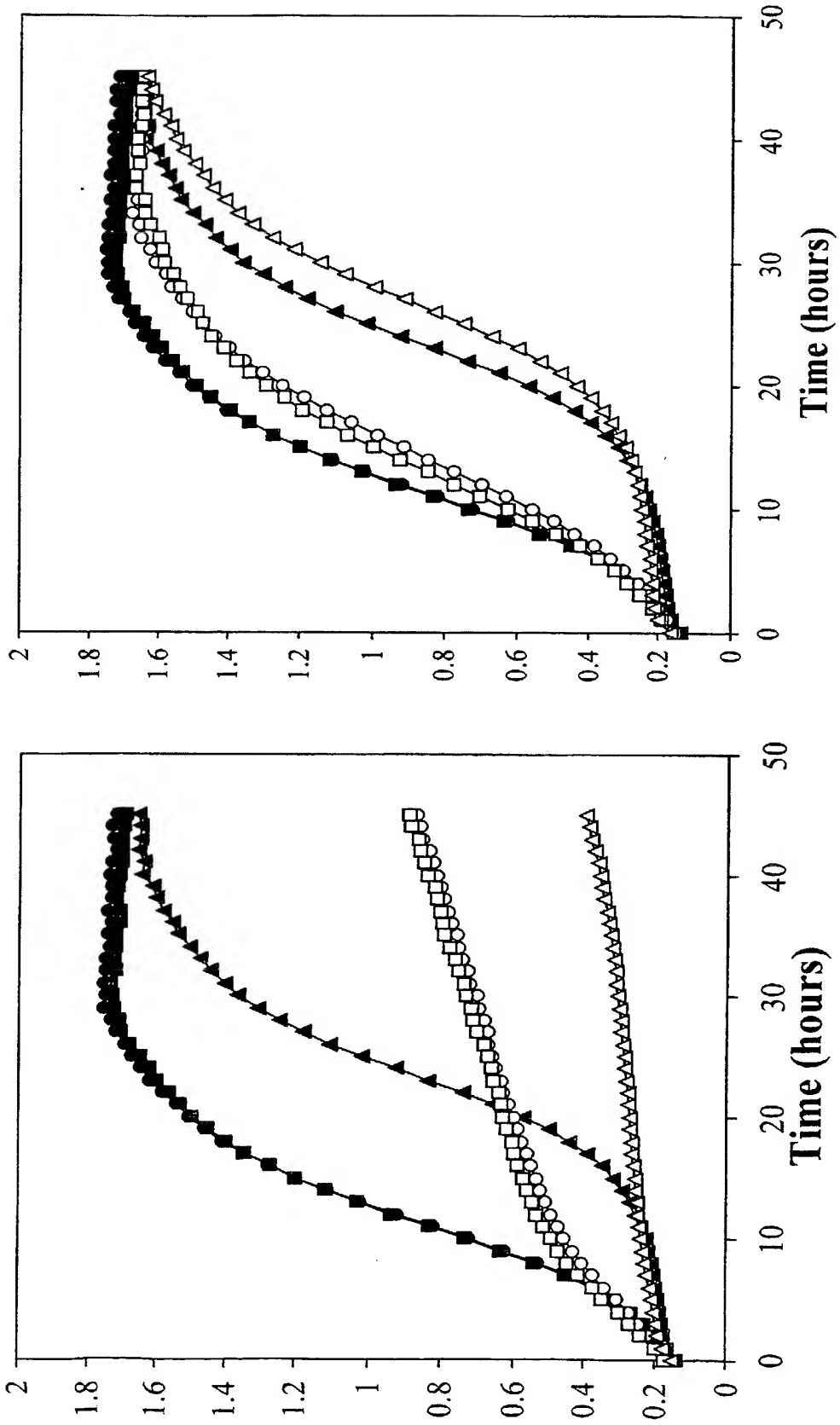
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
Time (hours)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
Time (hours)

Fig. 29

Docket No. 522-1780

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled Novel Target for Antiparasitic Agents and Inhibitors Thereof, the specification of which:

- is attached hereto.

X was filed on 08/29/00 as Application Serial No. PCT/EP00/08410
and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

PRIOR FOREIGN APPLICATION(S)

			Priority Claimed	
Country	**Number**	**Date Filed**	**Yes**	**No**
EP	99202805.0	August 30, 1999	___	___
EP	00870145.0	June 27, 2000	___	___

I hereby claim the benefit under Title 35, United States Code Section 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Section 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

Application Serial No.	**Filing Date**	**Status**
PCT/EP00/08410	08/29/2000	Pending

And I hereby appoint Robert F. I. Conte, Registration No. 20,354, Thomas E. Smith, Registration No. 18,243, Dennis M. McWilliams, Registration No. 25,195, James R. Sweeney, Registration No. 18,721, William M. Lee, Jr., Registration No. 26,935, Glenn W. Ohlson, Registration No. 28,455, David C. Brezina, Registration No. 34,128, Jeffrey R. Gray, Registration No. 33,391, Timothy J. Engling, Registration No. 39,970, Gerald S. Geren, Registration No. 24,528, Peter J. Shakula, Registration No. 40,808, John W. Hayes, Registration No. 33,900, Wm. Marshall Lee, Registration No. 16,853, Mark A. Hagedorn, Registration No. 44,731, Mark A. Nahnsen, Registration No. 51,093, to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith. It is requested that all communications be directed to:

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LEE, MANN, SMITH, MCWILLIAMS, SWEENEY & OHLSON
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Chicago, Illinois 60690-2786
telephone number (312) 368-1300
e-mail: mhagedorn@intelpro.com

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States

Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor: Johan Thevelein

Signature _____ Date 29 March 2002

Country of Residence: Belgium

Country of Citizenship: Belgium

Post Office and Residence Address: Groeneweg 46
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Full name of joint inventor: Patrick Van Dijck

Signature _____ Date 18/03/2002

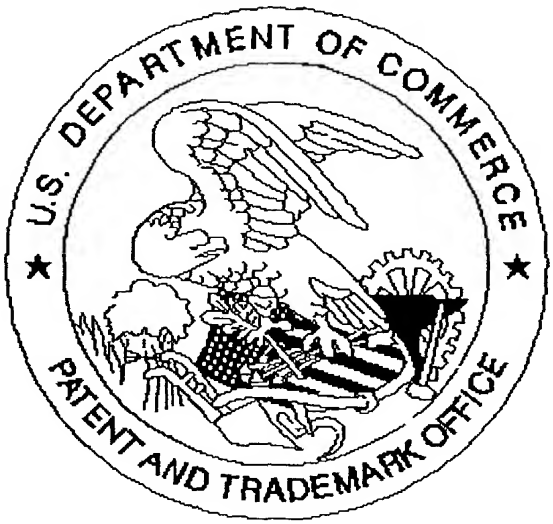
Country of Residence: Belgium

Country of Citizenship: Belgium

Post Office and Residence Address: Lobbensestraat 119
B-3271 Zichem, BELGIUM

United States Patent & Trademark Office

Office of Initial Patent Examination -- Scanning Division



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☐ Page(s)_____ of _____ were not present for scanning. (Document title)

☒ ***Scanned copy is best available.*** SOME DRAWINGS ARE DARK

[redacted]